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<b>(21) International Application Number:</b> PCT/DK97/00251 <b>(22) International Filing Date:</b> 6 June 1997 (06.06.97) <b>(30) Priority Data:</b> 08/660,289      7 June 1996 (07.06.96)      US <b>(71) Applicants:</b> NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsvaerd (DK). ZYMOGENETICS [US/US]; 1201 Eastlake Avenue East, Seattle, WA 98102 (US). <b>(72) Inventors:</b> PETERSEN, Lars, Christian; Havevej 4, DK-2970 Hørsholm (DK). HART, Charles, E.; 21502 21st Avenue West, Brier, WA 98036 (US). HEDNER, Ulla; Caritasgatan 19, S-216 18 Malmö (SE). RASMUSSEN, Mirella, Ezban; . . . Abildgaardsgade 24, DK-2100 Copenhagen Ø (DK). <b>(74) Common Representative:</b> NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsvaerd (DK).	<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>	
<b>(54) Title:</b> MODIFIED FACTOR VII  <b>(57) Abstract</b>  <p>The catalytic active site of Factor VII is modified to produce a compound which effectively interrupts the blood coagulation cascade. The modifications render Factor VIIa substantially unable to activate plasma Factors X or IX. The invention relates to novel methods of treatment and uses of modified Factor VII for treating preventing or treating myocardial injury associated with post-ischemic reperfusion, for improving regional myocardial blood flow during reperfusion, and maintaining or improving vascular patency in a patient, as well as topical application of modified Factor VII at vascular sites susceptible to thrombus formation.</p>		

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## MODIFIED FACTOR VII

Field of the Invention

The present invention relates to novel methods of treatment and novel uses of modified forms of Factor VII that inhibit thrombus formation, maintain or improve vascular patency, improve regional myocardial blood flow, and modulates myocardial injury during post-ischemic reperfusion.

Background Of The Invention

Blood coagulation is a process consisting of a complex interaction of various blood components, or factors, which eventually gives rise to a fibrin clot. Generally, the blood components which participate in what has been referred to as the coagulation "cascade" are proenzymes or zymogens, enzymatically inactive proteins which are converted to proteolytic enzymes by the action of an activator, itself an activated clotting factor. Coagulation factors that have undergone such a conversion and generally referred to as "active factors," and are designated by the addition of a lower case "a" suffix (e.g., Factor VIIa).

Activated Factor X ("Xa") is required to convert prothrombin to thrombin, which then converts fibrinogen to fibrin as a final stage in forming a fibrin clot. There are two systems, or pathways, that promote the activation of Factor X. The "intrinsic pathway" refers to those reactions that lead to thrombin formation through utilization of factors present only in plasma. A series of protease-mediated activations ultimately generates Factor IXa which, in conjunction with Factor VIIIa, cleaves Factor X into Xa. An identical proteolysis is effected by Factor VIIa and its co-factor, tissue factor, in the "extrinsic pathway" of blood coagulation. Tissue factor is a membrane bound protein and does not normally circulate in plasma. Upon vessel disruption, however, it can complex with Factor VIIa to catalyze Factor X activation or Factor IX activation in the presence of Ca<sup>++</sup> and phospholipid (Nemerson and Gentry, Biochem. 25:4020-4033 (1986)). While the relative importance of the two coagulation pathways in hemostasis is unclear, in recent years Factor VII and tissue factor have been found to play a pivotal role in the regulation of blood coagulation.

Factor VII is a trace plasma glycoprotein that circulates in blood as a single-chain zymogen. The zymogen is catalytically inactive (Williams et al., J. Biol. Chem. 264:7536-7543 (1989); Rao et al., Proc. Natl. Acad. Sci. USA. 85:6687-6691 (1988)). Single-chain

Factor VII may be converted to two-chain Factor VIIa by Factor Xa, Factor XIIa, Factor IXa or thrombin in vitro. Factor Xa is believed to be the major physiological activator of Factor VII. Like several other plasma proteins involved in hemostasis, Factor VII is dependent on vitamin K for its activity, which is required for the g-carboxylation of multiple glutamic acid  
5 residues that are clustered in the amino terminus of the protein. These g-carboxylated glutamic acids are required for the metal-associated interaction of Factor VII with phospholipids.

The conversion of zymogen Factor VII into the activated two-chain molecule occurs by cleavage of an internal peptide bond located approximately in the middle of the molecule.  
10 In human Factor VII, the activation cleavage site is at Arg<sub>152</sub>-Ile<sub>153</sub> (Hagen et al., Proc. Natl. Acad. Sci. USA 83: 2412-2416 (1986); Thim et al., Biochem. 27:7785-7793 (1988) both of which are incorporated herein by references). Bovine factor VII is activated by cleavage at the analogous Arg<sub>152</sub>-Ile<sub>153</sub> bond (Takeya et al., J. Biol. Chem. 263: 14868-14877, 1988). In the presence of tissue factor, phospholipids and calcium ions, the two-chain Factor VIIa  
15 rapidly activates Factor X or Factor IX by limited proteolysis.

It is often necessary to selectively block the coagulation cascade in a patient. Anticoagulants such as heparin, coumarin, derivatives of coumarin, indandione derivatives, or other agents may be used, for example, during kidney dialysis, or to treat deep vein thrombosis, disseminated intravascular coagulation (DIC), and a host of other medical  
20 disorders. For example, heparin treatment or extracorporeal treatment with citrate ion (U.S. Patent 4,500,309) may be used in dialysis to prevent coagulation during the course of treatment. Heparin is also used in preventing deep vein thrombosis in patients undergoing surgery.

Treatment with heparin and other anticoagulants may, however, have undesirable  
25 side effects. Available anticoagulants generally act throughout the body, rather than acting specifically at a clot site. Heparin, for example, may cause heavy bleeding. Furthermore, with a half-life of approximately 80 minutes, heparin is rapidly cleared from the blood, necessitating frequent administration. Because heparin acts as a cofactor for antithrombin III (AT III), and AT III is rapidly depleted in DIC treatment, it is often difficult to maintain the  
30 proper heparin dosage, necessitating continuous monitoring of AT III and heparin levels. Heparin is also ineffective if AT III depletion is extreme. Further, prolonged use of heparin

may also increase platelet aggregation and reduce platelet count, and has been implicated in the development of osteoporosis. Indandione derivatives may also have toxic side effects.

In addition to the anticoagulants briefly described above, several naturally occurring proteins have been found to have anticoagulant activity. For example, Reutelingsperger (U.S. Patent No. 4,736,018) isolated anticoagulant proteins from bovine aorta and human umbilical vein arteries. Maki et al. (U.S. Patent No. 4,732,891) disclose human placenta-derived anticoagulant proteins. In addition, AT III has been proposed as a therapeutic anticoagulant (Schipper et al., *Lancet* 1 (8069): 854-856 (1978); Jordan, U.S. Patent No. 4,386,025; Bock et al., U.S. Patent No. 4,517,294).

Proliferation of smooth muscle cells (SMCs) in the vessel wall is an important event in the formation of vascular lesions in atherosclerosis, after vascular reconstruction or in response to other vascular injury. For example, treatment of atherosclerosis frequently includes the clearing of blocked vessels by angioplasty, endarterectomy or reduction atherectomy, or by bypass grafting, surgical procedures in which atherosclerotic plaques are compressed or removed through catheterization (angioplasty), stripped away from the arterial wall through an incision (endarterectomy) or bypassed with natural or synthetic grafts. These procedures remove the vascular endothelium, disturb the underlying intimal layer, and result in the death of medial SMCs. This injury is followed by medial SMC proliferation and migration into the intima, which characteristically occurs within the first few weeks and up to six months after injury and stops when the overlying endothelial layer is reestablished. In humans, these lesions are composed of about 20% cells and 80% extracellular matrix.

In about 30% or more of patients treated by angioplasty, endarterectomy or bypass grafts, thrombosis and/or SMC proliferation in the intima causes re-occlusion of the vessel and consequent failure of the reconstructive surgery. This closure of the vessel subsequent to surgery is known as restenosis.

There is still a need in the art for improved compositions having anticoagulant activity which can be administered at relatively low doses and do not produce the undesirable side effects associated with traditional anticoagulant compositions. The present invention fulfills this need by providing anticoagulants that act specifically at sites of injury, and further provides other related advantages.

The modified Factor VII molecules are particularly useful for administration to humans to treat a variety of conditions involving intravascular coagulation. For example, although deep vein thrombosis and pulmonary embolism can be treated with conventional anticoagulants, the modified Factor VII described herein may be used to prevent the occurrence of

5 thromboembolic complications in identified high risk patients, such as those undergoing surgery or those with congestive heart failure. In addition, modified Factor VII may act as an antagonist for tissue factor-mediated induction of coagulation, thus blocking the production of thrombin and the subsequent deposition of fibrin. As such, modified Factor VII may be useful for inhibiting tissue factor activity resulting in, for example, the inhibition of blood

10 coagulation, thrombosis or platelet deposition.

The modified Factor VII molecules may be particularly useful in the treatment of intimal hyperplasia or restenosis due to acute vascular injury. Acute vascular injuries are those which occur rapidly (i.e. over days to months), in contrast to chronic vascular injuries (e.g. atherosclerosis) which develop over a lifetime. Acute vascular injuries often result from

15 surgical procedures such as vascular reconstruction, wherein the techniques of angioplasty, endarterectomy, atherectomy, vascular graft emplacement or the like are employed. Hyperplasia may also occur as a delayed response in response to, e.g., graft emplacement or organ transplantation. Since modified Factor VII is more selective than heparin, generally binding only tissue factor which has been exposed at sites of injury, and because modified

20 Factor VII does not destroy other coagulation proteins, it will be more effective and less likely to cause bleeding complications than heparin when used prophylactically for the prevention of deep vein thrombosis.

Recent advances in the treatment of coronary vascular disease include the use of mechanical interventions to either remove or displace offending plaque material in order to

25 re-establish adequate blood flow through the coronary arteries. Despite the use of multiple forms of mechanical interventions, including balloon angioplasty, reduction atherectomy, placement of vascular stents, laser therapy, or rotoblator, the effectiveness of these techniques remains limited by an approximately 40% restenosis rate within 6 months after treatment.

Restenosis is thought to result from a complex interaction of biological processes

30 including platelet deposition and thrombus formation, release of chemotactic and mitogenic factors, and the migration and proliferation of vascular smooth muscle cells into the intima of the dilated arterial segment.

The inhibition of platelet accumulation at sites of mechanical injury can limit the rate of restenosis in human subjects. Therapeutic use of a monoclonal antibody to platelet GpIIb/IIIa is able to limit the level of restenosis in human subjects (Califf et al., N. Engl. J. Med., 330:956-961 (1994)). The antibody is able to bind to the GpIIb/IIIa receptor on the surfaces of platelets and thereby inhibit platelet accumulation. This data suggests that inhibition of platelet accumulation at the site of mechanical injury in human coronary arteries is beneficial for the ultimate healing response that occurs. While platelet accumulation occurs at sites of acute vascular injuries, the generation of thrombin at these sites may be responsible for the activation of the platelets and their subsequent accumulation.

As shown in the examples that follow, the modified Factor VII of the present invention is able to bind to cell-surface tissue factor. For example, DEGR-Factor VIIa binds cell-surface tissue factor with an equivalent or higher affinity than wild-type Factor VIIa. DEGR-Factor VIIa, however, has no enzymatic activity, yet it binds to tissue factor and acts as a competitive antagonist for wild-type Factor VIIa, thereby inhibiting the subsequent steps in the extrinsic pathway of coagulation leading to the generation of thrombin.

Modified Factor VII molecules which maintain tissue factor binding inhibit platelet accumulation at the site of vascular injury by blocking the production of thrombin and the subsequent deposition of fibrin.

Due to the ability of DEGR-Factor VII to block thrombin generation and limit platelet deposition at sites of acute vascular injury, modified Factor VII molecules which maintain tissue factor binding activity but lack Factor VIIa enzymatic activity can be used to inhibit vascular restenosis.

Compositions comprising modified Factor VII are particularly useful in methods for treating patients when formulated into pharmaceutical compositions, where they may be given to individuals suffering from a variety of disease states to treat coagulation-related conditions. Such modified Factor VII molecules, capable of binding tissue factor but having a substantially reduced ability to catalyze activation of other factors in the clotting cascade, may possess a longer plasma half-life and thus a correspondingly longer period of anticoagulative activity when compared to other anticoagulants. Among the medical indications for the subject compositions are those commonly treated with anticoagulants, such as, for example, deep vein thrombosis, pulmonary embolism, stroke, disseminated intravascular coagulation (DIC), fibrin deposition in lungs and kidneys associated with gram-negative endotoxemia, and myocardial infarction. The compositions can be used to inhibit vascular restenosis as occurs following mechanical vascular injury, such as injury caused by balloon angioplasty, endarterectomy, reductive atherectomy, stent placement, laser therapy or rotablation, or as occurs secondary to vascular grafts, stents, bypass grafts or organ transplants. The compositions can thus be used to inhibit platelet deposition and associated disorders. Thus, a method of inhibiting coagulation, vascular restenosis or platelet deposition, for example, comprises administering to a patient a composition comprising modified Factor VII, such as that having at least one amino acid substitution in a catalytic triad of Ser<sub>344</sub>, Asp<sub>242</sub> and His<sub>193</sub>, in an amount sufficient to effectively inhibit coagulation, vascular restenosis or platelet deposition. The methods also find use in the treatment of acute closure of a coronary artery in an individual (e.g. acute myocardial infarction), which comprises administering the modified Factor VII, which includes DEGR-Factor VII and FFR-Factor VII, in conjunction with tissue plasminogen activator or streptokinase, and can accelerate tPA induced thrombolysis. The modified Factor VII is given prior to, in conjunction with, or shortly following administration of a thrombolytic agent, such as tissue plasminogen activator.

International Application No. WO 92/15686 relates to modified Factor VIIa, polynucleic acid and mammalian cell lines for the production of modified Factor VIIa, and compositions comprising modified Factor VIIa for inhibiting blood coagulation.

International Application No. WO 94/27631 relates to methods for inhibiting vascular restenosis, tissue factor activity, and platelet deposition.



International Application No. WO 96/12800 relates to a method for treatment of acute closure of a coronary artery comprising to the individual a composition which comprises modified Factor VIIa in conjunction with tissue plasminogen activator or streptokinase.

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#### Summary of the invention

The present invention relates to a method for inhibiting thrombus formation in a patient comprising administering topically to a vascular site susceptible to thrombus formation in the patient a therapeutically effective dose of a composition comprising Factor

10 VII having at least one modification in its catalytic center, which modification substantially inhibits the ability of the modified Factor VII to activate plasma Factor X or IX. The site of thrombus formation may be associated with surgery, microsurgery, angioplasty or trauma.

The invention further relates to a method for maintaining or improving vascular patency in a patient comprising administering locally to a vascular site susceptible to

15 decreased patency a therapeutically effective dose of a composition comprising Factor VII having at least one modification in its catalytic center, which modification substantially inhibits the ability of the modified Factor VII to activate plasma Factor X or IX. The site of thrombus formation may be associated with surgery, microsurgery, angioplasty or trauma.

The invention further relates to a method for preventing or minimizing myocardial

20 injury associated with post-ischemic reperfusion in an individual, comprising administering to the individual a composition which comprises a pharmacologically acceptable Factor VII having at least one modification in its catalytic center, which modification substantially inhibits the ability of the modified Factor VII to activate plasma Factor X or IX.

The invention further relates to a method for improving regional myocardial blood

25 flow during post-ischemic reperfusion in an individual, comprising administering to the individual a composition which comprises a pharmacologically acceptable Factor VII having at least one modification in its catalytic center, which modification substantially inhibits the ability of the modified Factor VII to activate plasma Factor X or IX.

In a preferred embodiment the modification of Factor VII comprises reaction of the

30 Factor VII with a serine protease inhibitor. In a more preferred aspect the protease inhibitor is an organophosphor compound, a sulfanyl fluoride, a peptide halomethyl ketone, or an azapeptide. In an even more preferred aspect the protease inhibitor is a peptide halomethyl

ketone selected from Dansyl-Phe-Pro-Arg chloromethyl ketone, Dansyl-Glu-Gly-Arg chloromethyl ketone, Dansyl-Phe-Phe-Arg chloromethyl ketone and Phe-Phe-Arg chloromethylketone, Phe-Phe-Arg chloromethylketone being the most preferred.

The invention further relates to the use of Factor VII having at least one modification  
5 in its catalytic center, which modification substantially inhibits the ability of the modified Factor VII to activate plasma Factor X or IX, for the manufacture of a composition for preventing or minimizing myocardial injury associated with post-ischemic reperfusion. The invention further relates to the use of Factor VII having at least one modification in its catalytic center, which modification substantially inhibits the ability of the modified Factor VII  
10 to activate plasma Factor X or IX, for the manufacture of a composition for improving regional myocardial blood flow during post-ischemic reperfusion.

The present invention provides methods and compositions to inhibit deleterious events associated with ischemic reperfusion. Severe ischemia to a tissue, organ or limb may be due to a decrease in blood flow and may be associated with trauma, surgical  
15 manipulation, or lowered blood pressure. One of the complications associated with severe ischemia is the up-regulation of tissue factor in the arterial system. This increased expression of tissue factor is believed to stimulate a procoagulant response, primarily in the capillary bed, thus initiating and/or sustaining intravascular thrombus formation. Furthermore, the *de novo* synthesis of TF during reperfusion of post-ischemic hearts by  
20 endothelial cells within the coronary vasculature may lead to a decrease in coronary blood flow during reperfusion and thus influencing the fate of the ischemic myocardium that will ultimately undergo necrosis. TF antigen and procoagulant activity is increased in atherectomy specimens obtained from patients with unstable angina, as compared to patients with stable angina. Thus, it is believed that in these patients unstable angina may be  
25 precipitated by exposure of TF in the subendothelial tissue of a large epicardial coronary artery as a result of plaque damage. This will eventually promote an intracoronary thrombus formation with a consequent absolute reduction in coronary flow.

TF may also effect coronary flow in a different way. Following reperfusion to the ischemic tissue, thrombi can be generated which may be either occlusive or non-occlusive.  
30 The generation of thrombi in the arterial bed, and the deposition of platelets along the thrombus, lead to the secondary generation of ischemia to the tissue. The generation of the thrombi and the presence of platelets can then cause the generation and release of multiple

bioactive factors, including those generated from the coagulation pathway, such as thrombin and Factor X, as well as factors released from activated platelets. In turn, these factors may induce the generation of additional factors by the underlying endothelial and smooth muscle cells, or by adjacent mononuclear cells, such as TNF-alpha and IL-1. These factors, in turn, can then activate the endothelial cells leading to the up-regulation of various adhesion molecules associated with monocyte and neutrophil binding. Normally, endothelial cells, being in contact with circulating blood, do not express significant TF activity. Under certain circumstances endothelial cells may actively promote coagulation by expressing TF-like procoagulant activity. In particular, both exogenous and endogenously generated oxygen free radicals (OFRs) can stimulate endothelial cells within the coronary vasculature to synthesize and express significant amounts of TF. OFRs are highly reactive molecular species that may attack various cell constituents. A burst of OFR generation follows restoration of flow after a period of ischemia, and these oxidant species might be responsible for a specific form of reperfusion-mediated tissue injury, secondary to lipid peroxidation and other irreversible alterations of cell constituents. OFRs also dramatically decrease the activity of tissue factor pathway inhibitor (TFPI), a Kunitz-type protein synthesized by endothelial cells, which inhibits the extrinsic coagulation pathway. This double effect of OFRs (TF expression by endothelial cells and decrease in TFPI activity) may shift the natural anticoagulant properties of the normal endothelium toward a procoagulant state, thus favouring an unwanted intravascular activation of the coagulation. Thus, OFR-mediated TF expression within the coronary circulation results in a significant reduction in coronary blood flow during post-ischemic reperfusion. This OFR-mediated expression of TF, with its attendant activation of the extrinsic coagulation pathway, has important consequences, as this phenomenon impacts on the pathophysiology of post-ischemic reperfusion, particularly in patients with acute myocardial infarction undergoing coronary thrombolysis.

The no-reflow phenomenon, that is, lack of uniform perfusion to the microvasculature of a previously ischemic tissue has been described for the first time by Krug et al., (Circ. Res. 1966; 19:57-62). The most important determinants that may influence the fate of ischemic myocardium are believed to be the amount of collateral flow during ischemia, the size of the area at risk and the myocardial oxygen demand.

Over the past decade there has been intense interest in the concept of treating patients with acute myocardial infarction with reperfusion strategies, including coronary throm-

bolysis, primary angioplasty, or both. However, not all studies have demonstrated an improvement in left ventricular function after recanalization of the infarct-related artery. At the moment, a substantial number of patients exhibit a "low-flow" condition in the infarct-related coronary bed. This condition is related with an almost complete lack of benefits at least in the term of mortality. These low-flow conditions is believed to be caused, at least in part, by the inability of blood to re-enter all of the vasculature of the previously ischemic myocardium. It has now surprisingly been shown that FVIIa effects, and increases, the regional myocardial blood flow during reperfusion. It has also surprisingly been shown that FVIIa results in a significant reduction in the area of no-reflow,

The binding and transmigration of monocytes and neutrophils, the release of bioactive compounds by these cells, including the generation of free-oxygen radicals, can exacerbate the level of endothelial cell activation and damage. Ultimately, if the cascade of events goes unchecked, this can lead to systemic complications and the potential to stimulate multiple organ failure. By blocking tissue factor by administering a specific inhibitor for tissue factor/Factor VII binding (e.g., FFR-FVIIa), and thereby blocking the initiation of the extrinsic pathway of coagulation, the initiation of the cascade of events may be prevented, thereby modulating the extent deleterious events associated with ischemia/reperfusion, such as, for example, eliminating, or minimizing the myocardial injury or necrosis.

#### Brief Description Of The Figures

Fig. 1 illustrates the construction of an expression vector for a Ser<sub>344</sub>Ala modified Factor VII DNA sequence. Symbols used include 0-1, the 0-1 map unit sequence from adenovirus 5; E, the SV40 enhancer; MLP, the adenovirus 2 major late promotor; SS, a set of splice sites; and pA, the polyadenylation signal from SV40 in the late orientation.

Fig. 2 shows the effect of bolus injection of DEGR-Factor VIIa on thrombus formation (platelet deposition) on endarterectomized baboon aorta when compared to saline-treated controls. The arteries were measured over 60 minutes. The DEGR-Factor VIIa significantly inhibited the development of platelet-rich thrombi in this primate model of acute vascular injury.

Fig. 3 shows results obtained when baboon smooth muscle cells were incubated with increasing concentrations of either FVIIa (open box), or DEGR-FVIIa in the presence of a constant amount of FVIIa (5 nM) (closed box). The level of FX activation was

subsequently determined using the chromogenic substrate S-2222. The data are presented as the amidolytic activity as a percentage of the activity generated in the presence of 5 nM FVIIa alone.

Fig. 4 depicts the size of the intimal area of baboons following carotid artery endarterectomy and treatment with DEGR-Factor VIIa for 7 or 30 days, compared to control animals.

Fig. 5 illustrates the ratio of the intimal area to the intimal + media area of baboon femoral artery following balloon injury and treatment with DEGR-Factor VIIa, where the control group included 5 vessels, 7 day treatment examined 11 vessels, and 30 day treatment examined 2 vessels (n= number of vessels examined).

Fig. 6 illustrates the experimental protocol for measuring infarct size (IS), no-reflow (NR), area at risk (AR), prothrombin time (PT) and activated partial thromboplastin time (aPTT).

Fig. 7 shows a plot of infarct size at the end of the reperfusion period (IS) expressed as a percent of the area at risk of infarcting in the three treatment groups, the three treatment groups being animals treated with FFR-Factor VIIa, Factor VIIa and saline, respectively. Each bar represents the mean of eight animals  $\pm$  SD.

Fig. 8 shows a plot of the area of no-reflow (NR) at the end of the reperfusion period expressed as a percent of the area at risk of infarcting. (animals treated with FFR-Factor VIIa, Factor VIIa and saline, respectively.) Each bar represents the mean of eight animals  $\pm$  SD.

Fig. 9 shows the relationship between expected no-reflow calculated (as a percent of left ventricle, LV) for each animal by the multiple linear regression equation and the no-reflow actually observed (as percent of LV).

Fig. 10 shows a plot of regional myocardial blood flow (RMBF) for ischemic myocardium assessed at 20 min of ischemia, and after 10 min and 2 hrs of reperfusion.

Fig. 11 shows the effect of FFR-Factor VIIa and Factor VIIa on prothrombin time (PT) and activated partial thromboplastin time (aPTT).

### Description Of The Specific Embodiments

Modified Factor VII can be in the form of the zymogen (i.e., a single-chain molecule) or can be cleaved at its activation site. Thus, by "modified Factor VII" is meant to include modified Factor VII and modified Factor VIIa molecules that bind tissue factor and inhibit the activation of Factor IX to IXa and Factor X to Xa. The Factor VII sequence has at least one amino acid modification, where the modification is selected so as to substantially reduce the ability of activated Factor VII to catalyze the activation of plasma Factors X or IX, and thus is capable of inhibiting clotting activity. The modified Factor VII has an active site modified by at least one amino acid substitution, and in its modified form is capable of binding tissue factor. The modified Factor VII compositions are typically in substantially pure form.

In preferred embodiments of human and bovine Factor VII, the active site residue Ser<sub>344</sub> is modified, replaced with Gly, Met, Thr, or more preferably, Ala. Such substitution could be made separately or in combination with substitution(s) at other sites in the catalytic triad, which includes His<sub>193</sub> and Asp<sub>242</sub>.

Compositions of the modified Factor VII are suitable for administration to a variety of mammals, particularly humans, to inhibit the coagulation cascade. Modified Factor VII may be administered to a patient in conjunction with or in place of other anticoagulant compounds. Typically, for administration to humans the pharmaceutical compositions will comprise modified human Factor VII protein and pharmaceutically-acceptable carriers and buffers.

Factor VII plays an important role in the coagulation cascade, particularly that involving the extrinsic pathway. Present in the circulating plasma as an inactive single chain zymogen protein, once activated, Factor VIIa, in combination with tissue factor and calcium ions, activates Factor X to Xa and activates Factor IX to IXa, with the eventual formation of a fibrin clot.

Factor VII proteins have a catalytic site which is modified to decrease the catalytic activity of Factor VIIa, while the molecule retains the ability to bind to tissue factor. The modified Factor VII molecules compete with native Factor VII and/or VIIa for binding to tissue factor. As a result, the activation of Factors X and IX is inhibited.

Modified Factor VII may be encoded by a polynucleotide molecule comprising two operatively linked sequence coding regions encoding, respectively, a pre-pro peptide and a gla domain of a vitamin K-dependent plasma protein, and a gla domain-less Factor VII

protein, wherein upon expression said polynucleotide encodes a modified Factor VII molecule which does not significantly activate plasma Factors X or IX, and is capable of binding tissue factor. The modified Factor VII molecule expressed by this polynucleotide is a biologically active anticoagulant, that is, it is capable of inhibiting the coagulation cascade and thus the formation of a fibrin deposit or clot. To express the modified Factor VII the polynucleotide molecule is transfected into mammalian cell lines, such as, for example, BHK, BHK 570 or 293 cell lines.

The catalytic activity of Factor VIIa can be inhibited by chemical derivatization of the catalytic center, or triad. Derivatization may be accomplished by reacting Factor VII with an irreversible inhibitor such as an organophosphor compound, a sulfonyl fluoride, a peptide halomethyl ketone or an azapeptide, or by acylation, for example. Preferred peptide halomethyl ketones include PPACK (D-Phe-Pro-Arg chloromethyl-ketone; (see U.S. Patent No. 4,318,904, incorporated herein by reference), D-Phe-Phe-Arg and Phe-Phe-Arg chloromethylketone (FFR-cmk); and DEGRck (dansyl-Glu-Gly-Arg chloromethylketone).

The catalytic activity of Factor VIIa can also be inhibited by substituting, inserting or deleting amino acids. In preferred embodiments amino acid substitutions are made in the amino acid sequence of the Factor VII catalytic triad, defined herein as the regions which contain the amino acids which contribute to the Factor VIIa catalytic site. The substitutions, insertions or deletions in the catalytic triad are generally at or adjacent to the amino acids which form the catalytic site. In the human and bovine Factor VII proteins, the amino acids which form a catalytic "triad" are Ser<sub>344</sub>, Asp<sub>242</sub>, and His<sub>193</sub> (subscript numbering indicating position in the sequence). The catalytic sites in Factor VII from other mammalian species may be determined using presently available techniques including, among others, protein isolation and amino acid sequence analysis. Catalytic sites may also be determined by aligning a sequence with the sequence of other serine proteases, particularly chymotrypsin, whose active site has been previously determined (Sigler et al., *J. Mol. Biol.*, 35:143-164 (1968), incorporated herein by reference), and therefrom determining from said alignment the analogous active site residues.

The amino acid substitutions, insertions or deletions are made so as to prevent or otherwise inhibit activation by the Factor VIIa of Factors X and/or IX. The Factor VII so modified should, however, also retain the ability to compete with authentic Factor VII and/or Factor VIIa for binding to tissue factor in the coagulation cascade. Such competition may

readily be determined by means of, e.g., a clotting assay as described herein, or a competition binding assay using, e.g., a cell line having cell-surface tissue factor, such as the human bladder carcinoma cell line J82 (Sakai et al. J. Biol. Chem. 264: 9980-9988 (1989), incorporated by reference herein.)

5           The amino acids which form the catalytic site in Factor VII, such as Ser<sub>344</sub>, Asp<sub>242</sub>, and His<sub>193</sub> in human and bovine Factor VII, may either be substituted or deleted. Within the present invention, it is preferred to change only a single amino acid, thus minimizing the likelihood of increasing the antigenicity of the molecule or inhibiting its ability to bind tissue factor, however two or more amino acid changes (substitutions, additions or deletions) may  
10   be made and combinations of substitution(s), addition(s) and deletion(s) may also be made. In a preferred embodiment for human and bovine Factor VII, Ser<sub>344</sub> is preferably substituted with Ala, but Gly, Met, Thr or other amino acids can be substituted. It is preferred to replace Asp with Glu and to replace His with Lys or Arg. In general, substitutions are chosen to disrupt the tertiary protein structure as little as possible. The model of Dayhoff et al. (in Atlas  
15   of Protein Structure 1978, Nat'l Biomed. Res. Found., Washington, D.C.), incorporated herein by reference, may be used as a guide in selecting other amino acid substitutions. One may introduce residue alterations as described above in the catalytic site of appropriate Factor VII sequence of human, bovine or other species and test the resulting protein for a desired level of inhibition of catalytic activity and resulting anticoagulant activity as described  
20   herein. For the modified Factor VII the catalytic activity will be substantially inhibited, generally less than about 5% of the catalytic activity of wild-type Factor VII of the corresponding species, more preferably less than about 1%.

          The modified Factor VII may be produced through the use of recombinant DNA techniques. In general, a cloned wild-type Factor VII DNA sequence is modified to encode  
25   the desired protein. This modified sequence is then inserted into an expression vector, which is in turn transformed or transfected into host cells. Higher eukaryotic cells, in particular cultured mammalian cells, are preferred as host cells. The complete nucleotide and amino acid sequences for human Factor VII are known. See U.S. Pat. No. 4,784,950, which is incorporated herein by reference, where the cloning and expression of recombinant  
30   human Factor VII is described. The bovine Factor VII sequence is described in Takeya et al., J. Biol. Chem. 263:14868-14872 (1988), which is incorporated by reference herein.



The amino acid sequence alterations may be accomplished by a variety of techniques. Modification of the DNA sequence may be by site-specific mutagenesis. Techniques for site-specific mutagenesis are well known in the art and are described by, for example, Zoller and Smith (DNA 3:479-488, 1984). Thus, using the nucleotide and amino acid sequences of Factor VII, one may introduce the alteration(s) of choice.

The Factor VII modified accordingly includes those proteins that have the amino-terminal portion (gla domain) substituted with a gla domain of one of the vitamin K-dependent plasma proteins Factor IX, Factor X, prothrombin, protein C, protein S or protein Z. The gla domains of the vitamin K-dependent plasma proteins are characterized by the presence of gamma-carboxy glutamic acid residues and are generally from about 30 to about 40 amino acids in length with C-termini corresponding to the positions of exon-intron boundaries in the respective genes. Methods for producing Factor VII with a heterologous gla domain are disclosed in U.S. Patent No. 4,784,950, incorporated by reference herein.

DNA sequences for use in producing modified Factor VII will typically encode a pre-pro peptide at the amino-terminus of the Factor VII protein to obtain proper post-translational processing (e.g. gamma-carboxylation of glutamic acid residues) and secretion from the host cell. The pre-pro peptide may be that of Factor VII or another vitamin K-dependent plasma protein, such as Factor IX, Factor X, prothrombin, protein C or protein S. As will be appreciated by those skilled in the art, additional modifications can be made in the amino acid sequence of the modified Factor VII where those modifications do not significantly impair the ability of the protein to act as an anticoagulant. For example, the Factor VII modified in the catalytic triad can also be modified in the activation cleavage site to inhibit the conversion of zymogen Factor VII into its activated two-chain form, as generally described in U.S. Patent 5,288,629, incorporated herein by reference.

Expression vectors for use in expressing modified Factor VIIa will comprise a promoter capable of directing the transcription of a cloned gene or cDNA. Preferred promoters for use in cultured mammalian cells include viral promoters and cellular promoters. Viral promoters include the SV40 promoter (Subramani et al., Mol. Cell. Biol. 1:854-864, 1981) and the CMV promoter (Boshart et al., Cell 41:521-530, 1985). A particularly preferred viral promoter is the major late promoter from adenovirus 2 (Kaufman and Sharp, Mol. Cell. Biol. 2:1304-1319, 1982). Cellular promoters include the mouse kappa gene promoter (Bergman et al., Proc. Natl. Acad. Sci. USA 81:7041-7045, 1983) and the

mouse  $V_H$  promoter (Loh et al., Cell 33:85-93, 1983). A particularly preferred cellular promoter is the mouse metallothionein-I promoter (Palmiter et al., Science 222:809-814, 1983). Expression vectors may also contain a set of RNA splice sites located downstream from the promoter and upstream from the insertion site for the Factor VII sequence itself.

- 5 Preferred RNA splice sites may be obtained from adenovirus and/or immunoglobulin genes. Also contained in the expression vectors is a polyadenylation signal located downstream of the insertion site. Particularly preferred polyadenylation signals include the early or late polyadenylation signal from SV40 (Kaufman and Sharp, *ibid.*), the polyadenylation signal from the adenovirus 5' Elb region, the human growth hormone gene terminator (DeNoto et al. Nuc. Acids Res. 9:3719-3730, 1981) or the polyadenylation signal from the human Factor VII
- 10 gene or the bovine Factor VII gene. The expression vectors may also include a noncoding viral leader sequence, such as the adenovirus 2 tripartite leader, located between the promoter and the RNA splice sites; and enhancer sequences, such as the SV40 enhancer.

- Cloned DNA sequences are introduced into cultured mammalian cells by, for
- 15 example, calcium phosphate-mediated transfection (Wigler et al., Cell 14:725-732, 1978; Corsaro and Pearson, Somatic Cell Genetics 7:603-616, 1981; Graham and Van der Eb, Virology 52d:456-467, 1973) or electroporation (Neumann et al., EMBO J. 1:841-845, 1982). To identify and select cells that express the exogenous DNA, a gene that confers a selectable phenotype (a selectable marker) is generally introduced into cells along with the
- 20 gene or cDNA of interest. Preferred selectable markers include genes that confer resistance to drugs such as neomycin, hygromycin, and methotrexate. The selectable marker may be an amplifiable selectable marker. A preferred amplifiable selectable marker is a dihydrofolate reductase (DHFR) sequence. Selectable markers are reviewed by Thilly (Mammalian Cell Technology, Butterworth Publishers, Stoneham, MA, incorporated herein
- 25 by reference). The choice of selectable markers is well within the level of ordinary skill in the art.

- Selectable markers may be introduced into the cell on a separate plasmid at the same time as the gene of interest, or they may be introduced on the same plasmid. If on the same plasmid, the selectable marker and the gene of interest may be under the control of
- 30 different promoters or the same promoter, the latter arrangement producing a dicistronic message. Constructs of this type are known in the art (for example, Levinson and

Simonsen, U.S. Patent 4,713,339). It may also be advantageous to add additional DNA, known as "carrier DNA," to the mixture that is introduced into the cells.

After the cells have taken up the DNA, they are grown in an appropriate growth medium, typically 1-2 days, to begin expressing the gene of interest. As used herein the  
5 term "appropriate growth medium" means a medium containing nutrients and other components required for the growth of cells and the expression of the modified Factor VII gene. Media generally include a carbon source, a nitrogen source, essential amino acids, essential sugars, vitamins, salts, phospholipids, protein and growth factors. For production of gamma-carboxylated modified Factor VII, the medium will contain vitamin K, preferably at  
10 a concentration of about 0.1 mg/ml to about 5 mg/ml. Drug selection is then applied to select for the growth of cells that are expressing the selectable marker in a stable fashion. For cells that have been transfected with an amplifiable selectable marker the drug concentration may be increased to select for an increased copy number of the cloned sequences, thereby increasing expression levels. Clones of stably transfected cells are then  
15 screened for expression of modified Factor VII.

Preferred mammalian cell lines include the COS-1 (ATCC CRL 1650), baby hamster kidney (BHK) and 293 (ATCC CRL 1573; Graham et al., J. Gen. Virol. 36:59-72, 1977) cell lines. A preferred BHK cell line is the tk<sup>-</sup> ts13 BHK cell line (Waechter and Baserga, Proc. Natl. Acad. Sci. USA 79:1106-1110, 1982, incorporated herein by reference), hereinafter  
20 referred to as BHK 570 cells. The BHK 570 cell line has been deposited with the American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD 20852, under ATCC accession number CRL 10314. A tk<sup>-</sup> ts13 BHK cell line is also available from the ATCC under accession number CRL 1632. In addition, a number of other cell lines may be used, including Rat Hep I (Rat hepatoma; ATCC CRL 1600), Rat Hep II (Rat hepatoma; ATCC  
25 CRL 1548), TCMK (ATCC CCL 139), Human lung (ATCC HB 8065), NCTC 1469 (ATCC CCL 9.1), CHO (ATCC CCL 61) and DUKX cells (Urlaub and Chasin, Proc. Natl. Acad. Sci. USA 77:4216-4220, 1980).

Transgenic animal technology may be employed to produce modified Factor VII. It is preferred to produce the proteins within the mammary glands of a host female mammal.  
30 Expression in the mammary gland and subsequent secretion of the protein of interest into the milk overcomes many difficulties encountered in isolating proteins from other sources. Milk is readily collected, available in large quantities, and well characterized biochemically.

Furthermore, the major milk proteins are present in milk at high concentrations (typically from about 1 to 15 g/l).

From a commercial point of view, it is clearly preferable to use as the host a species that has a large milk yield. While smaller animals such as mice and rats can be used (and are preferred at the proof of principle stage), it is preferred to use livestock mammals including, but not limited to, pigs, goats, sheep and cattle. Sheep are particularly preferred due to such factors as the previous history of transgenesis in this species, milk yield, cost and the ready availability of equipment for collecting sheep milk. See WIPO Publication WO 88/00239 for a comparison of factors influencing the choice of host species. It is generally desirable to select a breed of host animal that has been bred for dairy use, such as East Friesland sheep, or to introduce dairy stock by breeding of the transgenic line at a later date. In any event, animals of known, good health status should be used.

To obtain expression in the mammary gland, a transcription promoter from a milk protein gene is used. Milk protein genes include those genes encoding caseins (see U.S. Patent No. 5,304,489, incorporated herein by reference), beta-lactoglobulin, alpha-lactalbumin, and whey acidic protein. The beta-lactoglobulin (BLG) promoter is preferred. In the case of the ovine beta-lactoglobulin gene, a region of at least the proximal 406 bp of 5' flanking sequence of the gene will generally be used, although larger portions of the 5' flanking sequence, up to about 5 kbp, are preferred, such as a ~4.25 kbp DNA segment encompassing the 5' flanking promoter and non-coding portion of the beta-lactoglobulin gene. See Whitelaw et al., Biochem J. 286: 31-39 (1992). Similar fragments of promoter DNA from other species are also suitable.

Other regions of the beta-lactoglobulin gene may also be incorporated in constructs, as may genomic regions of the gene to be expressed. It is generally accepted in the art that constructs lacking introns, for example, express poorly in comparison with those that contain such DNA sequences (see Brinster et al., Proc. Natl. Acad. Sci. USA 85: 836-840 (1988); Palmiter et al., Proc. Natl. Acad. Sci. USA 88: 478-482 (1991); Whitelaw et al., Transgenic Res. 1: 3-13 (1991); WO 89/01343; and WO 91/02318, each of which is incorporated herein by reference). In this regard, it is generally preferred, where possible, to use genomic sequences containing all or some of the native introns of a gene encoding the protein or polypeptide of interest, thus the further inclusion of at least some introns from, e.g., the beta-lactoglobulin gene, is preferred. One such region is a DNA segment which provides for

intron splicing and RNA polyadenylation from the 3' non-coding region of the ovine beta-lactoglobulin gene. When substituted for the natural 3' non-coding sequences of a gene, this ovine beta-lactoglobulin segment can both enhance and stabilize expression levels of the protein or polypeptide of interest. Within other embodiments, the region surrounding the initiation ATG of the modified Factor VII sequence is replaced with corresponding sequences from a milk specific protein gene. Such replacement provides a putative tissue-specific initiation environment to enhance expression. It is convenient to replace the entire modified Factor VII pre-pro and 5' non-coding sequences with those of, for example, the BLG gene, although smaller regions may be replaced.

For expression of modified Factor VII in transgenic animals, a DNA segment encoding modified Factor VII is operably linked to additional DNA segments required for its expression to produce expression units. Such additional segments include the above-mentioned promoter, as well as sequences which provide for termination of transcription and polyadenylation of mRNA. The expression units will further include a DNA segment encoding a secretory signal sequence operably linked to the segment encoding modified Factor VII. The secretory signal sequence may be a native Factor VII secretory signal sequence or may be that of another protein, such as a milk protein. See, for example, von Heinje, Nuc. Acids Res. 14: 4683-4690 (1986); and Meade et al., U.S. Patent No. 4,873,316, which are incorporated herein by reference.

Construction of expression units for use in transgenic animals is conveniently carried out by inserting a modified Factor VII sequence into a plasmid or phage vector containing the additional DNA segments, although the expression unit may be constructed by essentially any sequence of ligations. It is particularly convenient to provide a vector containing a DNA segment encoding a milk protein and to replace the coding sequence for the milk protein with that of a modified Factor VII polypeptide, thereby creating a gene fusion that includes the expression control sequences of the milk protein gene. In any event, cloning of the expression units in plasmids or other vectors facilitates the amplification of the modified Factor VII sequence. Amplification is conveniently carried out in bacterial (e.g. *E. coli*) host cells, thus the vectors will typically include an origin of replication and a selectable marker functional in bacterial host cells.

The expression unit is then introduced into fertilized eggs (including early-stage embryos) of the chosen host species. Introduction of heterologous DNA can be

accomplished by one of several routes, including microinjection (e.g. U.S. Patent No. 4,873,191), retroviral infection (Jaenisch, Science 240: 1468-1474 (1988)) or site-directed integration using embryonic stem (ES) cells (reviewed by Bradley et al., Bio/Technology 10: 534-539 (1992)). The eggs are then implanted into the oviducts or uteri of pseudopregnant females and allowed to develop to term. Offspring carrying the introduced DNA in their germ line can pass the DNA on to their progeny in the normal, Mendelian fashion, allowing the development of transgenic herds.

General procedures for producing transgenic animals are known in the art. See, for example, Hogan et al., Manipulating the Mouse Embryo: A Laboratory Manual, Cold Spring Harbor Laboratory, 1986; Simons et al., Bio/Technology 6: 179-183 (1988); Wall et al., Biol. Reprod. 32: 645-651 (1985); Buhler et al., Bio/Technology 8: 140-143 (1990); Ebert et al., Bio/Technology 9: 835-838 (1991); Krimpenfort et al., Bio/Technology 9: 844-847 (1991); Wall et al., J. Cell. Biochem. 49: 113-120 (1992); U.S. Patent Nos. 4,873,191 and 4,873,316; WIPO publications WO 88/00239, WO 90/05188, WO 92/11757; and GB 87/00458, which are incorporated herein by reference. Techniques for introducing foreign DNA sequences into mammals and their germ cells were originally developed in the mouse. See, e.g., Gordon et al., Proc. Natl. Acad. Sci. USA 77: 7380-7384 (1980); Gordon and Ruddle, Science 214: 1244-1246 (1981); Palmiter and Brinster, Cell 41: 343-345 (1985); Brinster et al., Proc. Natl. Acad. Sci. USA 82: 4438-4442 (1985); and Hogan et al. (*ibid.*). These techniques were subsequently adapted for use with larger animals, including livestock species (see e.g., WIPO publications WO 88/00239, WO 90/05188, and WO 92/11757; and Simons et al., Bio/Technology 6: 179-183 (1988)). To summarize, in the most efficient route used to date in the generation of transgenic mice or livestock, several hundred linear molecules of the DNA of interest are injected into one of the pro-nuclei of a fertilized egg according to established techniques. Injection of DNA into the cytoplasm of a zygote can also be employed.

Production in transgenic plants may also be employed. Expression may be generalized or directed to a particular organ, such as a tuber. See, Hiatt, Nature 344:469-479 (1990); Edelbaum et al., J. Interferon Res. 12:449-453 (1992); Sijmons et al., Bio/Technology 8:217-221 (1990); and European Patent Office Publication EP 255,378.

Modified Factor VII may be purified by affinity chromatography on an anti-Factor VII antibody column. The use of calcium-dependent monoclonal antibodies, as described by

Wakabayashi et al., J. Biol. Chem. 261:11097-11108, (1986) and Thim et al., Biochem. 27: 7785-7793, (1988), incorporated by reference herein, is particularly preferred. Additional purification may be achieved by conventional chemical purification means, such as high performance liquid chromatography. Other methods of purification, including barium citrate precipitation, are known in the art, and may be applied to the purification of the novel modified Factor VII described herein (see, generally, Scopes, R., Protein Purification, Springer-Verlag, N.Y., 1982). Substantially pure modified Factor VII of at least about 90 to 95% homogeneity is preferred, and 98 to 99% or more homogeneity most preferred, for pharmaceutical uses. Once purified, partially or to homogeneity as desired, the modified Factor VII may then be used therapeutically.

The modified Factor VII is cleaved at its activation site to convert it to its two-chain form. Activation may be carried out according to procedures known in the art, such as those disclosed by Osterud, et al., Biochemistry 11:2853-2857 (1972); Thomas, U.S. Patent No. 4,456,591; Hedner and Kisiel, J. Clin. Invest. 71:1836-1841 (1983); or Kisiel and Fujikawa, Behring Inst. Mitt. 73:29-42 (1983), which are incorporated herein by reference. The resulting molecule is then formulated and administered as described below.

### Compositions

The compounds will typically be administered within about 24 hours prior to performing the intervention, and for as much as 7 days or more thereafter. Administration for preventing or minimizing myocardial injury can be by a variety of routes as further described herein. The compounds can also be administered locally at vascular sites susceptible of thrombus formation, for example, at sites of anastomosis, or locally at vascular sites susceptible to decreased patency.

In the prevention of or treatment of myocardial injury, the dose of modified Factor VII ranges from about 50 mg to 500 mg/day, more typically 1 mg to 200 mg/day, and more preferably 10 mg to about 175 mg/day for a 70 kg patient as loading and maintenance doses, depending on the weight of the patient and the severity of the condition.

The pharmaceutical compositions for treatment of myocardial injuries are intended for parenteral administration for prophylactic and/or therapeutic treatment. Preferably, the pharmaceutical compositions are administered parenterally, i.e., intravenously, subcutaneously, or intramuscularly. The compositions for parenteral administration

comprise a solution of the modified Factor VII molecules dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine and the like. The modified Factor VII molecules can also be formulated into liposome preparations for delivery or targeting to sites of injury.

5 Liposome preparations are generally described in, e.g., U.S. 4,837,028, U.S. 4,501,728, and U.S. 4,975,282, incorporated herein by reference. The compositions may be sterilized by conventional, well known sterilization techniques. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The

10 compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, etc. The concentration of modified Factor VII in these formulations can vary widely, i.e., from less than about 0.5%, usually at or at least about 1%

15 to as much as 15 or 20% by weight and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

Thus, a typical pharmaceutical composition for intravenous infusion could be made up to contain 250 ml of sterile Ringer's solution, and 10 mg of modified Factor VII. Actual methods for preparing parenterally administrable compounds will be known or apparent to

20 those skilled in the art and are described in more detail in for example, Remington's Pharmaceutical Science, 16th ed., Mack Publishing Company, Easton, PA (1982), which is incorporated herein by reference.

The compositions containing the modified Factor VII molecules can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, compositions are

25 administered to a patient already suffering from a disease, as described above, in an amount sufficient to cure or at least partially arrest the disease and its complications. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend on the severity of the disease or injury and the weight and general state of the patient, but generally range from about 0.05 mg up to about 500 mg of modified

30 Factor VII per day for a 70 kg patient, with dosages of from about 1.0 mg to about 200 mg of modified Factor VII per day being more commonly used. It must be kept in mind that the materials of the present invention may generally be employed in serious disease or injury



states, that is, life-threatening or potentially life threatening situations. In such cases, in view of the minimization of extraneous substances and general lack of immunogenicity of modified human Factor VII in humans, it is possible and may be felt desirable by the treating physician to administer substantial excesses of these modified Factor VII compositions.

5           In prophylactic applications, compositions containing the modified Factor VII are administered to a patient susceptible to or otherwise at risk of a disease state or injury to enhance the patient's own anticoagulative capabilities. Such an amount is defined to be a "prophylactically effective dose." In this use, the precise amounts again depend on the patient's state of health and weight, but generally range from about 0.05 mg to about 500 mg  
10       per 70 kilogram patient, more commonly from about 1.0 mg to about 200 mg per 70 kg of body weight.

          Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. For ambulatory patients requiring daily maintenance levels, the modified Factor VII may be administered by  
15       continuous infusion using a portable pump system, for example.

          Local delivery of the modified Factor VII such as, for example topical application of modified Factor VII at vascular sites susceptible to thrombus formation, (e.g. sites of anastomosis) or at vascular sites susceptible to decreased patency may be carried out, for example, by way of spray, perfusion, double balloon catheters, stent, incorporated into  
20       vascular grafts or stents, hydrogels used to coat balloon catheters, or other well established methods. In any event, the pharmaceutical formulations should provide a quantity of modified Factor VII of this invention sufficient to effectively treat the patient.

          The following examples are offered by way of illustration, not by way of limitation.

## EXAMPLES

EXAMPLE IExpression of Ser<sub>344</sub>®Ala<sub>344</sub> Factor VII

5 To generate the Ser<sub>344</sub>®Ala Factor VII active site mutant, plasmid FVII(565+2463)/pDX (U.S. Patent No. 4,784,950 incorporated herein by reference; deposited with the American Type Culture Collection under accession number 40205) was digested with Xba I and Kpn I, and the resulting 0.6 kb fragment, comprising the coding region for serine 344, was recovered. This fragment was cloned into Xba I, Kpn I-digested M13mp19 as shown in  
10 the Figure. This manipulation and subsequent steps described below were generally performed according to standard protocols (as described, for example, by Maniatis et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1982) incorporated herein by reference).

Mutagenesis was carried out on the M13 template according to the methods of  
15 Zoller and Smith, supra, using the mutagenic oligonucleotide ZC1656 (5' TGG GCC TCC GGC GTC CCC CTT 3') and the "universal" second primer ZC87 (5' TCC CAG TCA CGA CGT 3'). Reaction products were screened using kinased ZC1656. Positive plaques were picked, and template DNA was prepared and sequenced from the Pst I site at 1077 to the Kpn I site at 1213. Sequence analysis confirmed the presence of the desired mutation. The  
20 mutant clone was designated 1656.

An expression vector was then constructed using the 1656 clone. The mutagenized sequence was isolated from the M13 vector as a ~0.14 kb Pst I-Kpn I fragment. This fragment was ligated to the 1.7 kb Hind III-Xba I fragment from FVII(565+2463)/pDX, the 0.5 kb Xba I-Pst I fragment from FVII(565+2463)/pDX, and the 4.3 kb Kpn I-Hind III fragment  
25 from FVII(565+2463)/pDX, as shown in the Figure. The presence of the desired mutant sequence was confirmed by digesting mutant and wild-type clones with Pst I, and a mutant Factor VII insert in M13 with Kpn I and Xba I, preparing Southern blots of the digested DNA, and probing the blots with radiolabeled ZC1656.

The baby hamster kidney cell line BHK 570 (deposited with the American Type  
30 Culture Collection under accession number 10314) was transfected with two isolates (designated #544 and #545) of the 1656 expression vector. The cells were prepared by diluting a confluent 10 cm plate of BHK 570 cells 1:10 into five 10 cm plates in non-selective

medium (Dulbecco's modified Eagle's medium [DMEM] containing 10% fetal bovine serum and 1% PSN antibiotic mix [GIBCO Life Technologies, Gaithersburg, MD]). After 24 hours, when the cells had reached 20-30% confluency, they were co-transfected with one isolate of the expression vector encoding the 1656 mutation, plasmid p486 (comprising the Adenovirus 5 ori, SV40 enhancer, Adenovirus 2 major late promotor, Adenovirus 2 tripartite leader, 5' and 3' splice sites, the DHFR' cDNA and SV40 polyadenylation signal in pML-1 (Lusky and Botchan, *Nature* 293: 79-81, (1981)) and 10 mg of carrier DNA (sonicated salmon sperm DNA) as shown in Table 1. The DNA was added to a 15 ml tube, then 0.5 ml of 2X Hepes (25 g Hepes, 40 g NaCl, 1.8 g KCl, 0.75 g Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, 5 g dextrose diluted to 2.5 l with distilled water and pH adjusted to pH 6.95-7.0) was added and the tubes were mixed. The DNA in each tube was precipitated by the addition of 0.5 ml of 0.25 M CaCl<sub>2</sub> while air was bubbled through the DNA/Hepes solution with a pasteur pipet. The tubes were then vortexed, incubated at room temperature for 15 minutes, and vortexed again. The DNA mixtures were then added dropwise onto the plates of cells with a pipette. The plates were swirled and incubated at 37°C for 4-6 hours. After incubation, 2 ml of 20% glycerol diluted in Tris-saline (0.375 g KCl, 0.71 g Na<sub>2</sub>HPO<sub>4</sub>, 8.1 g NaCl, 3.0 g Tris-HCl, 0.5 g sucrose, diluted in a total of 1 liter and pH adjusted to pH 7.9) was then added to each plate. The plates were swirled and left at room temperature for two minutes. The medium was then removed from the plates and replaced with 2 ml of Tris-saline. The plates were left at room temperature for 2 minutes, then the Tris-saline was removed and replaced with 10 ml of non-selective medium. The plates were incubated at 37°C for two days.

Table 1  
Transfection\*

	544	545	544 Control	545 Control
Plasmid Name				
Clone 544	15 ml	---	15 ml	---
Clone 545	---	30 ml	---	30 ml
p486	1.5 ml	1.5 ml	---	---
Carrier DNA	1.6 ml	1.6 ml	1.6 ml	1.6 ml

- 5 \* DNA concentrations used were: clone 544: 0.7 mg/ml; clone 545: 0.3 mg/ml; p486: 1.49 mg/ml.

After the two day incubation, the cells were diluted in selection medium (DMEM containing 10% dialyzed fetal bovine serum, 1% PSN antibiotic mix and 150 nM  
10 methotrexate) and plated at dilutions of 1:100, 1:250 and 1:500 in maxi plates. The plates were incubated at 37°C for one week. After one week, the medium was changed and replaced with selection medium, and the plates were checked for colony formation.

Eight days later, after colony formation, twelve colonies were randomly chosen from the 1:500 dilution plates of the #544 and #545 transfection plates. Each clone was plated  
15 into one well of a 6-well plate and grown in selection medium. After seven days, the plates were confluent, and the clones were each split into 10 cm plates in selection medium.

The clones described above and control cells transfected to express wild-type factor VII were metabolically labeled with <sup>35</sup>S-Methionine-Cysteine Protein Labeling Mix (NEN DuPont Biotechnology Systems, Wilmington, DE). The clones were grown and prepared for  
20 a pulse label experiment in selective medium. The cells were rinsed with phosphate buffered saline (Sigma, St. Louis, MO) and pulsed for four hours in 20 mCi/ml <sup>35</sup>S-Cys-<sup>35</sup>S-Met. After four hours, supernatants and cells were harvested. The cells were lysed essentially as described by Lenk and Penman (Cell 16: 289-302, (1979)) and 400 ml of each lysate and precleared with 50 ml of staph A (Sigma, St. Louis, MO).

5        Samples from the metabolically labeled cells were radioimmunoprecipitated (RIP) by first incubating the samples with 6 ml of anti-Factor VII polyclonal antisera for four hours. Sixty microliters of washed staphylococcal protein A was added to each sample, and the samples were rocked for 1.5 hours at 4°C. The samples were centrifuged, and the supernatant was removed. The pellets were washed twice in 0.7 M RIPA buffer (10 mM Tris, pH 7.4, 1% deoxycholic acid [Calbiochem Corp., La Jolla, CA], 1% Triton X-100, 0.1% SDS, 5 mM EDTA, 0.7 M NaCl) and once in 0.15 M RIPA buffer (10 mM Tris, pH 7.4, 1% deoxycholic acid [Calbiochem Corp., La Jolla, CA], 1% Triton X-100, 0.1 SDS, 5 mM EDTA, 0.15 M NaCl). One hundred microliters of 1x SDS dye (50 mM Tris-HCl, pH 6.8, 100 mM  
10        dithiothreitol, 2% SDS, 0.1% bromphenol blue, 10% glycerol) was added to each sample, and the samples were boiled for 5 minutes followed by centrifugation to remove the protein A. Fifty microliters of each sample was run on a 10% polyacrylamide gel. Results showed that 9 of 10 clones secreted modified Factor VII.

## 15        EXAMPLE II

### ANTICOAGULANT ACTIVITY OF MODIFIED FACTOR VII

      The ability of the modified Factor VII protein to inhibit clotting was measured in a one-stage clotting assay using wild-type Factor VII as a control. Recombinant proteins were prepared essentially as described above from cells cultured in media containing 5mg/ml  
20        vitamin K. Varying amounts of the modified Factor VII (from clone 544) or recombinant wild-type Factor VII were diluted in 50 mM Tris pH 7.5, 0.1% BSA to 100 ml. The mixtures were incubated with 100 ml of Factor VII-deficient plasma (George King Bio-Medical Inc., Overland Park, KS) and 200 ml of thromboplastin C (Dade, Miami, FL; contains rabbit brain thromboplastin and 11.8 mM Ca<sup>++</sup>). The clotting assay was performed in an automatic  
25        coagulation timer (MLA Electra 800, Medical Laboratory Automation Inc., Pleasantville, NY), and clotting times were converted to units of Factor VII activity using a standard curve constructed with 1:5 to 1:640 dilutions of normal pooled human plasma (assumed to contain one unit per ml Factor VII activity; prepared by pooling citrated serum from healthy donors). Using this assay the preparations of modified Factor VII exhibited no detectable coagulant  
30        activity. Table 2 shows results of the assay in terms of clotting times for control (untransfected) BHK cell-conditioned media (+/- vitamin K), wild-type Factor VII and two

isolates of cells expressing the modified Factor VII. Factor VII activity is seen as a reduction in clotting time over control samples.

Table 2

Sample	Dilution	Clotting Time (sec.)
Control +K	1:5	33.1
	1:10	33.4
Control -K	1:5	34.3
	1:10	33.2
Wild-type Factor VII	1:20	19.0
	1:40	21.5
	1:80	23.3
Modified Factor VII (#6)	1:1	33.5
Modified Factor VII (#10)	1:1	32.5

5

To determine the effect of the modified Factor VII on plasma factor substrates, preparations of modified Factor VII and recombinant wild-type or native Factor VII are incubated with either Factor X or Factor IX and the activation thereof monitored by clotting assays or polyacrylamide gel electrophoresis.

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### **EXAMPLE III**

#### **Ability of Modified Factor VII to Bind Tissue Factor**

The ability of the modified Factor VII to compete with wild-type Factor VII for tissue factor and inhibit its clotting activity was assessed in a one-step clotting assay in the presence of a limiting amount of tissue factor (thromboplastin).

Clotting times were determined in a one-step assay similar to that described in Example II. A limited amount of tissue factor, a constant amount of wild type Factor VII, and increasing amounts of variant Factor VII were used in the mixing experiments. An inhibition of Factor VII/VIIa procoagulant activity would be seen as an increase in clotting time in assays containing increasing amounts of variant Factor VII.

20

The amount of Factor VII activity in the test samples was calculated as a percentage of a standard curve that measured Factor VII activity in normal pooled plasma. The standard curve for Factor VII activity was generated using serial dilutions of normal pooled plasma in phosphate buffered solution (PBS) that ranged from 1:5 to 1:640. For this purpose it was assumed that normal plasma contains approximately 500 ng/ml of Factor VII and this was considered to be one unit of activity. A mixture of 100 ml Factor VII-deficient plasma, 100 ml plasma dilution and 200 ml of thromboplastin-C (Dade, Miami, FL.) was used to measure clotting time on a MLA Electra 800 automatic timer. To establish the standard curve, the results were graphed as percentage of activity (1:5 = 100% activity) versus clotting time in seconds.

The assay required that the medium containing the wild type and variant Factor VII be composed of less than one percent serum. The dilutions were made in PBS so that clotting times would fall along the standard curve. A minimum dilution of 1:2 was typical. The final volume was 100 ml. Two different human Factor VII Ser<sub>344</sub>® Ala variants, designated clones "#10" and "#6" were tested in the experiments. The results, set forth in the Table below, show that as the amount of Factor VII variant increased, the percent of Factor VIIa activity decreased.

Table 3:

Results of mixing assay with Ser344 -&gt; Ala

Variants (B4A1 (wild type) medium was used as 100% activity at 10 µl/reaction)

5

Ser344 -> Ala Clone No.	Variant medium amount	B4A1 medium amount	BHK control*	Percent FVIIa Activity
#10	10 µl	10 µl	0	70
#10	20 µl	10 µl	0	51
#10	30 µl	10 µl	0	43
#10	40 µl	10 µl	0	34
#10	50 µl	10 µl	0	28
#10 (-K) <sup>§</sup>	20 µl	10 µl	0	78
#6	10 µl	10 µl	0	74
#6	20 µl	10 µl	0	56
#6	30 µl	10 µl	0	46
#6	40 µl	10 µl	0	41
#6	50 µl	10 µl	0	32
#6	20 µl	10 µl	0	85
BHK control	0	10 µl	20 µl	91
BHK control (-K)	0	10 µl	20 µl	107

\* Untransfected conditioned medium

<sup>§</sup> For expression of the Factor VII variant, cells were grown in the presence of vitamin K, except where noted "(-K)".

10

These experiments showed that variants of Factor VII having a Ser<sub>344</sub> ® Ala substitution competed with native Factor VII in a dose dependent fashion and inhibited the procoagulant activity of native Factor VII/VIIa. It can thus be concluded that Ser<sub>344</sub> ® Ala variant human Factor VII competes with native human Factor VIIa and consequently inhibits

15

activation of Factor X and/or IX in human plasma.



#### EXAMPLE IV

##### Reaction of Factor VII with PPACK

5           Recombinant Factor VII was produced in transfected baby hamster kidney cells. The protein was purified and activated as disclosed by Thim et al. (Biochemistry 27: 7785-7793, 1988), Brinkous et al. (Proc. Natl. Acad. Sci. USA 86: 1382-1386, 1989) and Bjoern and Thim (Res. Discl. No. 269, 564, 1986), which are incorporated herein by reference. The cell culture medium was recovered, filtered and diluted to reduce salt concentration. The  
10   diluted medium was then fractionated by anion exchange chromatography using an elution buffer containing  $\text{CaCl}_2$ . The Factor VII fraction was recovered and further purified by immunochromatography using a calcium-dependent anti-Factor VII monoclonal antibody. Additional purification was carried out using two anion exchange chromatography steps wherein Factor VII was eluted using  $\text{CaCl}_2$  and NaCl, respectively. Factor VIIa was  
15   recovered in the final eluate.

          Recombinant Factor VIIa (1 mM) in 50 mM Tris-HCl, 100 mM NaCl, 5 mM  $\text{CaCl}_2$ , pH 7.4 was incubated with 20 mM PPACK (D-Phenylalanyl-Prolyl-Arginyl Chloromethyl Ketone; Calbiochem, La Jolla, CA) for 5, 20 and 60 minutes. Buffer containing the chromogenic substrate S2288 (H-D-Isoleucine-L-Prolyl-L-Arginine p-nitroanilide; Kabi Vitrum AB, Molndal,  
20   Sweden) was then added to obtain a 2.5 fold dilution and a final concentration of 0.3 mM S2288. The generation of p-nitroaniline was measured and compared to results using untreated Factor VIIa as a control. The results indicated that Factor VIIa is fully inactivated after about 60 minutes under these reaction conditions.

25

#### EXAMPLE V

##### Generation of DEGR-Factor VIIa

          Recombinant human Factor VIIa was prepared as described in Example IV. Recombinant human Factor VIIa, in 10 mM glycine buffer, pH 8.0, 10 mM  $\text{CaCl}_2$ , 50 mM  
30   NaCl, was diluted to a concentration of 1.5 mg/ml. A 10-fold molar excess of Dansyl-L-Glu-Gly-Arg-Chloromethyl Ketone, DEGRck, (Calbiochem, La Jolla, CA 92037) which had been dissolved with distilled  $\text{H}_2\text{O}$  was added to the Factor VIIa. After a 2 hr incubation at 37°C, a

second 10-fold molar excess of DEGRck was added to the mixture and incubated for an additional 2 hr at 37°C. A third 10-fold molar excess of DEGRck was added to the Factor VIIa and incubated for approximately 16 hours at 4°C. The DEGR-Factor VIIa sample was then extensively dialyzed at 4°C against Tris buffered saline (0.05 M Tris-HCl, 0.1 M NaCl, pH 7.5) to remove any free DEGRck.

The final DEGR-Factor VIIa mixture was tested for the presence of free DEGRck in a Factor Xa chromogenic substrate assay. The DEGR-Factor VIIa mixture was added to purified human Factor Xa along with the chromogenic substrate S-2222. This substrate is cleaved specifically by Factor Xa and not by Factor VIIa. Unbound DEGRck in the mixture is able to bind to the Factor Xa and thereby inhibit the chromogenic activity of the Factor Xa. Spiking free DEGR-ck into a Factor Xa mixture generated a standard curve to measure the level of free DEGRck in solution versus the inhibition of Factor Xa chromogenic activity. Analysis of the DEGR-Factor VIIa mixture showed that the ratio of free DEGRck:DEGR-Factor VIIa was less than 0.5% following extensive dialysis, thereby ensuring that the inhibition observed by DEGR-Factor VIIa in the various assay systems described below was not due to the presence of free DEGRck.

#### **EXAMPLE VI**

##### **Factor Xa Generation on Rat Smooth Muscle Cells**

Vascular smooth muscle cells were analyzed for the presence of cell-surface tissue factor by measuring the ability of the cells to stimulate the conversion of Factor X to Factor Xa using a chromogenic substrate that is specific for Factor Xa.

Rat vascular smooth muscle cells (Clowes et al., J. Clin. Invest. 93:644-651 (1994)) were plated into 96-well culture dishes (American Scientific Products, Chicago, IL.) at 8,000 cells per well in growth media (Table 4).

Table 4

- 500 ml Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO-BRL, Gaithersburg, MD.)  
 10% fetal calf serum (Hyclone, Logan, UT.)  
 5 1mM sodium pyruvate (Irvine, Santa Ana, CA.)  
 0.29 mg/ml L-glutamine (Hazelton, Lenexa, KS.)  
 1x PSN; (100X is 5 mg/ml penicillin, 5 mg/ml streptomycin,  
 10 mg/ml neomycin) (GIBCO-BRL, Gaithersburg, MD.)
- 10 After a 48 hour incubation at 37°C the medium was changed to serum free medium (Table 5).

Table 5

- 250 ml Dulbecco's Modified Eagle's Medium (DMEM)  
 15 250 ml Ham's F-12 Medium (Fred Hutchinson Cancer Research Center, Seattle, WA)  
 1mM sodium pyruvate  
 .29 mg/ml L-glutamine  
 20 mM transferrin (JRH, Lenexa, KS.)  
 5 mM insulin (GIBCO-BRL)  
 20 16 ng selenium (Aldrich, Milwaukee, WI.)  
 1 mg/ml bovine serum albumin (Sigma, St. Louis, MO)

- The cells were incubated 72 hours at 37°C. After incubation, either PDGF-BB (10 ng/ml) or 10% fetal calf serum was added to the cells to stimulate tissue factor  
 25 expression (Taubman et al., *J. Clin. Invest.* 91:547-552, 1993). A parallel set of cells received neither PDGF nor serum to monitor for intrinsic activity of unstimulated cells. After a 6 hour incubation, recombinant human Factor VIIa was added to the cells at a final concentration of 10 nM. One set of cells did not have Factor VIIa added as a negative control. The cells were incubated for 2 hours at 37°C and washed with HEPES buffer (10  
 30 mM HEPES, 137 mM NaCl, 4 mM KCl, 5 mM CaCl<sub>2</sub>, 11 mM glucose, 0.1% BSA). After washing, cells were incubated for 5 min with 50 µl per well of 200 nM plasma-purified human Factor X in a Tris-buffered saline supplemented with 5 mM CaCl<sub>2</sub>. Twenty-five

microliters of 0.5 M EDTA and 25 ml of an 800 mM solution of S-2222 chromogenic substrate (Kabi Pharmacia, Franklin, OH) were added to each well. The plates were incubated for 40 min at room temperature, then analyzed at 405 nm using a THERMOMAX microplate reader (Molecular Devices, Menlo Park, CA).

5        Table 6 shows an increase in absorbance for the Factor VIIa treated wells as compared to the control wells (no Factor VIIa added). The increase in absorbance is a direct measurement of the level of Factor Xa generated in the wells and its subsequent cleavage of the chromogenic substrate, releasing the chromophore. The data also demonstrate that the level of chromogenic activity in cells pretreated with either PDGF-BB or 10% fetal calf serum  
10        was higher than unstimulated cells.

Table 6

Test Sample	OD <sub>405</sub>
Control	0.043
Intrinsic	0.247
PDGF-BB	0.360
10% FCS	0.342

These results clearly show there is a Factor VIIa-dependent activation of Factor X to  
15        Factor Xa on the cell surface of rat vascular smooth muscle cells.

#### EXAMPLE VII

##### Inhibition of Cell-Surface Chromogenic Activity By DEGR-Factor VIIa

20        Rat vascular smooth muscle cells were plated into 96-well culture dishes as described above. The cells were cultured for 72 hours in serum free media as described above and treated with the addition of 10% fetal calf serum for 6 hours to stimulate tissue factor expression. After stimulation, buffer only (control), 10 nM Factor VIIa, or 10 nM Factor VIIa + 100 nM DEGR-Factor VIIa was added to each well. The cells were incubated for 2  
25        hours at 37°C, then washed with HEPES buffer. After washing, the cells were incubated for 5 minutes with 50 ml per well of 200 nM Factor X in Tris-buffered saline supplemented with 5

mM CaCl<sub>2</sub>. Twenty-five microliters of 0.5 M EDTA and 25 ml of S-2222 (800 mM) chromogenic substrate (Kabi Pharmacia) were added to each well. The cells were incubated at room temperature for 40 minutes. Chromogenic activity was analyzed at 405 nm as described above.

5            Table 7 shows stimulation of chromogenic activity in the wells treated with Factor VIIa only, and inhibition of stimulation when DEGR-Factor VIIa was co-incubated with the Factor VIIa. These results demonstrate that DEGR-Factor VIIa acts as a competitive antagonist for Factor VIIa binding, thereby inhibiting the activation of Factor X to Factor Xa and the subsequent cleavage of the S-2222 chromogen.

10

Table 7

Test Sample	OD <sub>405</sub>
Control	0.035
Factor VIIa	0.342
Factor VIIa + DEGR-Factor VIIa	0.073

#### EXAMPLE VIII

15    Dose Dependent Inhibition by DEGR-Factor VIIa of Cell Surface Chromogenic Activity on Rat Smooth Muscle Cells

Rat vascular smooth muscle cells were plated into 96-well culture dishes at 4,000 cells per well in growth medium supplemented with 1% fetal calf serum (as in Table 4 without 10% fetal calf serum). After 5 days the medium was removed, and either increasing  
20    concentrations of Factor VIIa alone or 10 nM Factor VIIa with increasing concentrations of DEGR-Factor VIIa were added to the cells. The cells were incubated with the Factor VII mixtures for 2 hours at 37°C. After incubation, the cells were washed and incubated with 50 ml of 200 nM Factor X in tris buffered saline for 5 minutes at room temperature. Each well had 25 ml of 0.5 M EDTA and 25 ml of 800 mM S-2222 (Kabi Pharmacia) added to it, and  
25    the plates were incubated for 40 minutes at room temperature. Chromogenic activity was analyzed at 405 nm in a microplate reader as described above.

Table 8 shows a dose-dependent increase in chromogenic activity with increasing amounts of Factor VIIa added to the wells. When the mixture of DEGR-Factor VIIa with 100 nM Factor VIIa was added to the cells (Table 9) there was a dose dependent inhibition in chromogenic activity. A 1:1 molar ratio of DEGR-Factor VIIa:Factor VIIa inhibited approximately 95% of the chromogenic activity. These data suggest that in this experimental design DEGR-Factor VIIa has a significantly higher affinity for cell-surface tissue factor than native Factor VIIa on smooth muscle cells in culture. If DEGR-Factor VIIa and Factor VIIa had equal affinity for binding tissue factor then the level of inhibition observed when the two molecules were added to the cells in an equal molar ratio would not have been as high.

Table 8

Factor VIIa Conc. (nM)	OD <sub>405</sub>
0.10	0.005
0.39	0.025
1.56	0.058
6.25	0.111
25.00	0.154
100.00	0.208

Table 9 shows the dose dependent inhibition of Factor Xa chromogenic activity on rat smooth muscle cells by DEGR-Factor VIIa. Increasing concentrations of DEGR-Factor VIIa were co-incubated with 100 nM Factor VIIa, and the Factor Xa chromogenic activity determined using chromogenic substrate S-2222.

Table 9

DEGR-Factor VIIa Conc. (nM)	OD <sub>405</sub>
0.10	0.208
0.39	0.176
1.56	0.116
6.25	0.073
25.00	0.026
100.00	0.014

## 5 **EXAMPLE IX**

### Inhibition of Factor Xa generation by DEGR-Factor VIIa in a soluble tissue factor assay.

The conversion of Factor X to Factor Xa using purified recombinant soluble tissue factor was established using a chromogenic assay. Tissue factor was expressed and purified from *Saccharomyces cerevisiae* (Shigematsu et al., *J. Biol. Chem.* 267:21329-21337, 1992). Soluble tissue factor was purified and characterized by Dr. W. Kisiel (University of New Mexico). A reaction mixture containing 65.9 ml of soluble tissue factor (2.2 mM), 29.0 ml of PCPS (1 mM, Sigma, St. Louis, MO), 29.5 ml human Factor X (4.1 mM), 2.77 ml Hank's buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 2.7 mM KCl, 5 mM CaCl<sub>2</sub>, 0.1% BSA) was prepared. Forty microliter of tissue factor/Factor X mixture, 25 ml Factor VIIa diluted with TBS and 25 ml of DEGR-Factor VIIa diluted with TBS were added to each well of a 96-well microtiter plate. A control using 40 ml of tissue factor/Factor X mixture; 25 ml Factor VIIa diluted with TBS, and 25 ml of TBS only was included. Ten microliters of S-2222 (4 mM) chromogenic substrate was added to the reaction mixture in the wells and incubated at room temperature for 2-10 minutes. Results were analyzed at 405 nm in a microplate reader as described above.

Determination of a standard curve for Factor VIIa activation of Factor X was made using increasing concentrations of Factor VIIa added in the absence of DEGR-Factor VIIa. The results, presented in Table 10, show that there is a dose-dependent increase in

chromogenic activity with increasing amounts of Factor VIIa added to the reaction mixture. The simultaneous addition of varying amounts of DEGR-Factor VIIa and 100 nM Factor VIIa led to a dose dependent decrease in chromogenic activity (Table 11). These data demonstrate that DEGR-Factor VIIa acts as a competitive antagonist for native Factor VIIa binding to soluble tissue factor, and thereby inhibits the generation of Factor Xa as measured by the decrease in chromogenic activity towards the chromogenic substrate S-2222.

Table 10

- 10 Stimulation of Factor Xa chromogenic activity with increasing concentrations of Factor VIIa added to soluble tissue factor. Changes in optical density were measured using chromogenic substrate S-2222.

Factor VIIa Conc (nM)	OD <sub>405</sub>
0.78	0.168
1.56	0.288
3.12	0.478
6.25	0.694
12.50	0.764
25.00	0.790
50.00	0.738
100.00	0.770



Table 11

Inhibition of Factor Xa chromogenic activity by the addition of DEGR-Factor VIIa to soluble tissue factor in the presence of native Factor VIIa is measured. Changes in optical density were measured using the chromogenic substrate S-2222.

DEGR-Factor VIIa Conc. (nM)	OD <sub>405</sub>
0	0.810
50	0.750
100	0.609
200	0.296
400	0.167
800	0.083
1600	0.055

#### EXAMPLE X

##### 10 Inhibition of Coagulation by DEGR-Factor VIIa

Standard clotting assays to monitor the effect of DEGR-Factor VIIa on clotting time were prepared as follows: 100 ml of normal baboon plasma, collected with sodium citrate as anticoagulant, was added to 100 ml of varying concentrations of DEGR-Factor VIIa diluted in TBS (20 mM Tris, pH 7.4, 150 mM NaCl). The samples were mixed and briefly incubated at 15 37°C. The samples were added to an Electra 800 Automatic Coagulation Timer (Medical Laboratories Automation, Pleasantville, NY). After incubation, 200 ml of a tissue factor preparation containing 25 mM CaCl<sub>2</sub> was added to the DEGR-Factor VIIa preparations. A tissue factor preparation was made as a saline extract of baboon brain from freshly frozen brain tissue and characterized for its ability to initiate coagulation in baboon plasma. A 20 concentration of tissue factor that gave a clotting time of about 40 seconds was selected.

The data, presented in Table 12, demonstrates a dose-dependent increase in clotting time due to the addition of DEGR-Factor VIIa. A dose as low as 1 mg/ml of DEGR-Factor VIIa in plasma resulted in a significant increase in clotting time.

5

Table 12

Dose dependent increase in clotting time due to DEGR-Factor VIIa.

DEGR-Factor VIIa ( $\mu$ g/ml plasma)	Clotting Time (seconds)
0	40.7
0.5	46.2
1.0	50.8
2.5	64.5
5.0	108.1
10.0	158.4

10 **EXAMPLE XI****Inhibition of Platelet Accumulation With DEGR-Factor VIIa**

DEGR-Factor VIIa was analyzed for its ability to inhibit platelet accumulation at sites of arterial thrombosis due to mechanical injury in non-human primates. A model of aortic endarterectomy was utilized in baboons, essentially as described by Lumsden et al. (Blood 15 81:1762-1770 (1993)). A section of baboon aorta 1-2 cm in length was removed, inverted and scraped to remove the intima of the artery and approximately 50% of the media. The artery was reverted back to its correct orientation, cannulated on both ends and placed into an extracorporeal shunt in a baboon, thereby exposing the mechanically injured artery to baboon blood via the shunt. Just prior to opening of the shunt to the circulating blood, <sup>111</sup>In- 20 labeled autologous platelets were injected intravenously into the animal. The level of platelet accumulation at the site of the injured artery was determined by real-time gamma camera imaging.

Evaluation of DEGR-Factor VIIa for inhibition of platelet accumulation was done using bolus injections of DEGR-Factor VIIa or saline control and were given just prior to the opening of the shunt. The injured arteries were measured continuously for 60 minutes. A dose of 0.005 mg/kg of DEGR-Factor VIIa inhibited platelet accumulation. At a 1.0 mg/kg bolus injection, approximately 90% of platelet accumulation was inhibited at 1 hour post drug administration. These results are shown in Fig. 2.

These data show that inhibition of tissue factor with DEGR-Factor VIIa can significantly inhibit the development of platelet-rich thrombi in a nonhuman primate model of acute vascular injury.

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#### EXAMPLE XII

##### DEGR-FVIIa Inhibits Vascular Restenosis Following Balloon Angioplasty in Atherosclerotic

##### Rabbits

DEGR-FVIIa was evaluated for its ability to modulate lesion development following balloon angioplasty in New Zealand White (NZW) atherosclerotic rabbits. This animal model has been well characterized and has proven to be a good model for evaluating anti-thrombotic compounds on vascular lesion development (Gimple et al., Circulation 86:1536-1546 (1992), and Rogosta et al., Circulation 89:1262-1271 (1994)). The animal model used to evaluate DEGR-FVIIa is essentially as described by Ragosta, ibid.

Anesthesia was induced in rabbits with 5 mg/kg xylazine and 35 mg/kg ketamine by intramuscular injection. The proximal femoral arteries were exposed by cutdown below the inguinal ligament with proximal and distal ligatures. The isolated segments were cannulated with 27 gauge needles. A vent was created by needle puncture. The isolated segments were flushed with saline to clear residual blood, and desiccated by air infused at a rate of 80 ml/min for 8 minutes. Following air-drying, the isolated segments were again flushed with saline and the ligatures removed. Hemostasis was maintained with non-occlusive local pressure. The segments were demarcated with metal clips. Local spasm was treated with Xylocaine 1% locally. The day following surgery, the animals were placed on 1% cholesterol and 6% peanut oil diet for one month until balloon angioplasty. Tylenol 10 mg/kg orally was

given for postoperative pain relief for 3-5 days. Ambipen 1cc was given after the surgical procedure during postoperative days 3 to 5.

The test drug delivery for the animals consisted of an initial bolus injection immediately prior to balloon angioplasty, followed by a continuous systemic infusion by osmotic pump via the internal jugular vein. The duration of the drug infusion was 3 days. The control animals received heparin, 150 U/kg IV bolus, prior to balloon angioplasty followed by saline infusion. The DEGR-FVIIa treated animals received a 1 mg/kg bolus injection followed by 50 mg/kg/hr infusion.

For the placement of the osmotic pumps for continuous systemic infusion, anesthesia was induced in the animals as described above, and maintained throughout the procedure with additional IM injections of ketamine and Xylazine. Through a midline neck incision, the right internal jugular vein was isolated by blunt dissection and the distal end ligated. A silastic tube (PE-160) was introduced into the right internal jugular vein. A subcutaneous tunnel was created to pass the silastic tube. This tube was connected with the osmotic pump. The osmotic pump was implanted subcutaneously in the back of the rabbit. The right common carotid artery was isolated by blunt dissection and the distal end ligated. Via an arteriotomy, a 5F introducer was placed and advanced to the junction of the aortic arch. Blood was drawn for determination of hemostatic parameters, drugs and cholesterol levels. Twenty milligrams of xylocain was injected intraarterially. A control aortoiliiofemoral angiogram was performed via a 5F Berman catheter positioned above the aortic bifurcation using 3-4 ml renographin injected over 3 seconds by hand.

After removal of the Berman catheter, a 0.014-inch guidewire was introduced in the descending aorta and positioned above the aortic bifurcation. Under fluoroscopic guidance, an appropriately sized balloon angioplasty catheter of 2.0 to 2.5 mm was introduced and advanced over the guidewire and positioned across the stenosis. The balloon was inflated to 6 atmospheres for 60 seconds with a hand inflator. Three inflations were performed with 60 second intervals between inflations. This procedure was performed in both femoral arteries in each animal.

Following balloon dilatation, the angioplasty catheter was withdrawn and the Berman catheter reintroduced to a position 3 cm above the aortic bifurcation. To minimize spasm 20 mg of lidocaine was given intraarterially. A post procedure angiogram was performed as described above. A 1 cm grid was positioned at the level of the femoral artery to calculate

the actual diameter. The catheter was then removed. The right carotid artery was ligated with 3-0 silk and the wound sutured by layers. Ambipen and acetaminophen were given as above.

Prothrombin time and concentration of DEGR-FVIIa in the blood were determined at immediately pre-bolus injection of the test compound, 1 hr post bolus injection, and at 3 days at the end of continuous infusion. One to two mls of citrated plasma was obtained and the prothrombin times and antigen levels determined.

A standard clotting assay was used to monitor the prothrombin time in the control and DEGR-FVIIa-treated animals as follows. Twenty-five microliters of test rabbit plasma, collected with sodium citrate as anticoagulant, was added to 150 ml of TBS (20 mM Tris, pH 7.4, 150 mM NaCl). The samples were mixed and added to an Electra 800 Automated Coagulation Timer (Medical Laboratories Automation, Pleasantville, NY). After incubation, 200 ml of thromboplastin preparation (Sigma Chemical) containing 25 mM  $\text{CaCl}_2$  was added to the plasma preparations. A concentration of thromboplastin that gave a clotting time of approximately 20 seconds in the control rabbit plasma was selected.

An ELISA assay was used to determine the concentration of DEGR-FVIIa in plasma samples from the control and DEGR-FVIIa treated rabbits. The assay involved first diluting an anti-human FVII monoclonal antibody (Dr. W. Kisiel, U. of New Mexico) to 2.0 mg/ml in 0.1 M carbonate buffer pH 9.6, and adding 100 ml/well to 96-well plates. The plates were then incubated at 4°C overnight and subsequently washed two times using wash buffer (PBS, pH 7.4, containing 0.05% Tween 20). Blocking of nonspecific binding sites was achieved with 200 ml of blocking buffer per well (PBS, pH 7.4, containing 0.05% Tween 20 and 1% BSA) incubated at 37°C for 2 hr, followed by a wash using the wash buffer.

After blocking, a standard dilution series of DEGR-FVIIa ranging from 20-0.027 ng/ml was added, along with a dilution series of the test rabbit plasma (1:100 to 1:4000 in blocking buffer) applied at 100 ml/well. Non-immune rabbit plasma was used as a negative control. Plates were then incubated for 1 hr at 37°C followed by four washes with wash buffer.

DEGR-FVIIa was detected by adding 100 ml/well of a 1:1,000 dilution of rabbit anti-human FVII polyclonal antibody (Dr. Kisiel, U. New Mexico) in blocking buffer. Plates were incubated for 1 hr at 37°C, followed by five washes with wash buffer. Specific antibody binding was detected using 100 ml/ well of a 1:2,000 dilution of goat anti-rabbit IgG antibody-

peroxidase conjugate (Tago, Inc.). Plates were incubated for 1 hr at 37°C and washed six times with wash buffer. Finally, 100 µl of substrate solution was added (0.42 mg/ml of o-phenylenediamine dihydrochloride [OPD] in 0.2 M citrate buffer, pH 5.0, containing 0.3% H<sub>2</sub>O<sub>2</sub>). After 1-3 min at room temp. the color reaction was stopped by adding 100 µl/well of 5 1N H<sub>2</sub>SO<sub>4</sub> and the plates were read at 490nm on a Microplate spectrophotometer. The concentration of DEGR-FVIIa in the plasma samples was determined by comparing the A<sub>490</sub> values of the unknown to those of the DEGR-FVIIa standard curve.

Analysis of plasma samples for prothrombin times and DEGR-FVIIa antigen levels is shown in Table 13 and Table 14, respectively. The data are presented for each individual 10 animal. Table 15 shows a summary of the mean clotting times. In all cases, the DEGR-FVIIa treated animals had elevated prothrombin times at the 1 hr post-bolus injection time point which returned to near pre-treatment levels at the 3-day time point. Analysis of the DEGR-FVIIa antigen levels also showed a high level of DEGR-FVIIa in the plasma at the 1 hr time point, ranging between 2-6 mg/ml in the plasma, with much lower circulating levels 15 at the 3 day time point. The levels of DEGR-FVIIa measured at the 1 hr time period correspond with a predicted increase in prothrombin time, as determined by spiking normal rabbit plasma with DEGR-FVIIa *in vitro* and determining prothrombin times in a standard dilute thromboplastin assay.

Table 13 MEASUREMENT OF PROTHROMBIN TIMES				
		Clotting Time (seconds)		
Animal Number	Treatment	Pretreatment	1 hour	3 days
73	Control	24.8	22.3	17.8
74	Control	24.8	27.9	18.6
75	Control	24.6	N/D	20.5
76	Control	22	N/D	17.9
169	Control	21.2	22.9	22
170	Control	24.9	23.5	18.6
173	Control	25.9	21	20.8
174	Control	25	29.4	20.1
77	DEGR-FVIIa	22.5	40.1	18.3
78	DEGR-FVIIa	24.3	34	20.9
80	DEGR-FVIIa	24.7	50	21.7
96	DEGR-FVIIa	N/A	N/A	21
97	DEGR-FVIIa	23.6	33.3	21.2
171	DEGR-FVIIa	20.6	45.8	21.9
172	DEGR-FVIIa	23.5	41.6	22.4

N/A = Data Not Available

Table 14  
ELISA TO DETECT DEGR-FVIIa IN RABBIT PLASMA

		FVIIa ELISA (ng/ml)		
Animal Number	Treatment	Pretreatment	1 hour	3 days
73	Control	0	13	0
74	Control	36	14	4
75	Control	0	N/A	9
76	Control	0	N/A	14
169	Control	0	0	1
170	Control	0	0	0
173	Control	36	31	0
174	Control	87	86	160
77	DEGR-FVIIa	0	3,210	102
78	DEGR-FVIIa	1	4,950	7
80	DEGR-FVIIa	13	4,543	661
96	DEGR-FVIIa	65	4,900	117
97	DEGR-FVIIa	4	4,600	502
171	DEGR-FVIIa	13	2,145	212
172	DEGR-FVIIa	9	2,830	228

N/A = Data Not Available



## 5 Table 15. Statistical Summary of Plasma Clotting Times.

## Unpaired t-Test X

DF:

12

Unpaired t Value: . . .

1.12

PRE-BLEED

Prob. (2-tail):

0.2852

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	8	24.15	1.64	0.58
DEGR-VIIa	6	23.2	1.48	0.60

10

## Unpaired t-Test X

DF:

10

Unpaired t Value:

-5.44

1 Hr POST ANGIO

Prob. (2-tail):

0.0003

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	6	24.5	3.35	1.37
DEGR-VIIa	6	40.8	6.53	2.67

## Unpaired t-Test X

DF:

13

Unpaired t Value:

-2.04

3 Days POST ANGIO

Prob. (2-tail):

0.0622

Group:	Count:	Mean:	Std. Dev.:	Std. Error:
Control	8	19.54	1.53	0.54
DEGR-VIIa	7	21.06	1.33	0.50

Three weeks post-angioplasty a follow-up angiogram was repeated as described above via the left carotid artery immediately prior to sacrifice. Through a vertical lower abdominal incision, the distal aorta was isolated, tied off proximally, and the perfusion cannula inserted above the aortic bifurcation. The distal aorta was flushed with 50 ml of saline followed by in vivo fixation with 500 ml of Histochoice (AMRESCO, Solon, OH) solution infused over 15 mins at 120 mmHg. Once perfusion was started, the animals were sacrificed with an overdose of nembutal (3 ml sodium pentobarbital IV, 65 mg/ml). A 5 cm segment of femoral artery was excised bilaterally. The tissue was preserved in Histochoice solution for light microscopy.

To determine intimal lesion development at the site of balloon angioplasty, the excised femoral arteries were cut in serial 3 mm sections, embedded in paraffin, and sections cut from multiple regions of each artery. The sections were mounted onto glass slides and the slides stained with hematoxylin and eosin, and Van Gieson stains.

Morphometric analysis was performed with Bioquant Program to obtain area measurements for the lumen, the intima and the media. Morphometric analysis of tissue sections from the injured arteries were done measuring the total luminal area; the area of the intima, determined by measuring the area within the internal elastic lamina and subtracting the corresponding luminal area from each tissue section; and the area of the media, determined by measuring the area inside the external elastic lamina and subtracting the area inside the internal elastic lamina. Measurements for intimal lesions in the femoral arteries in control and DEGR-FVIIa treated animals showed that there was a significant decrease in the size of the intima in the DEGR-FVIIa treated animals (Table 16). In contrast, measurement of the medial area showed no significant difference between the two groups.

Table 16. MEASUREMENTS OF THE INTIMA AND MEDIA IN BALLOON ANGIOPLASTY TREATED RABBITS				
Group	N	Intima (mm <sup>2</sup> )	Std. Dev.	Prob. (2-tail)
Control	13	0.819	0.414	0.0138
DEGR-FVIIa	10	0.438	0.192	
Group	N	Media (mm <sup>2</sup> )	Std. Dev.	Prob. (2-tail)
Control	13	0.389	0.098	0.172
DEGR-FVIIa	10	0.329	0.105	

The data from the angiographic measurements are presented in Table 17 as the

5 Mean Luminal Diameter (MLD) +/- standard deviation for the control and DEGR-FVIIa  
treated animal for all three time points: immediately pre-angioplasty, immediately  
post-angioplasty, and 21 days post-angioplasty. There was no significant difference in the  
MLD between the control and DEGR-FVIIa treated animals at either the pre- or immediately  
post-angioplasty measurements. A significant increase in MLD was observed, however, in  
10 the DEGR-FVIIa treated animals at the 21 day post-angioplasty measurement.

Table 17. MEASUREMENT OF MINIMAL LUMINAL DIAMETER (MLD)				
Pre-PTCA Measurement of MLD				
Group	N	Mean MLD	Std. Dev.	Prob. (2-tail)
Control	13	1.202	0.24	0.3883
DEGR-FVIIa	10	1.283	0.19	
Post-PTCA Measurement of MLD				
Group	N	Meal MLD	Std. Dev.	Prob. (2-tail)
Control	13	1.492	0.551	0.5326
DEGR-FVIIa	10	1.323	0.725	
21 Day Measurement of MLD				
Group	N	Mean MLD	Std. Dev.	Prob. (2-tail)
Control	13	0.889	0.228	0.0001
DEGR-FVIIa	10	1.393	0.242	

### 5 EXAMPLE XIII

#### Inhibition of Cell-Surface Factor Xa Generation on Baboon SMCs by DEGR-FVIIa

A cell-surface chromogenic assay was developed, essentially as described in  
 10 Example VIII above, to measure the efficacy of DEGR-FVIIa to block FVIIa binding to

cell-surface tissue factor and the subsequent conversion of Factor X to Factor Xa on monolayers of baboon smooth muscle cells (SMCs). This method is a modification of those described by Sakai et al., J. Bio. Chem. 264:9980-9988 (1989) and Wildgoose et al., Proc. Natl. Acad. Sci. USA. 87:7290-7294 (1990). Baboon SMCs were obtained from the

5 University of Washington, Seattle, WA, and were cultured from aortic explants. The baboon SMCs were plated into 96-well culture dishes at a concentration of 8,000 cells/well in 200 ml/well DMEM culture media supplemented with 10% fetal calf serum, and maintained in this media for 4 days at 37°C in 5% CO<sub>2</sub>. At the time of assay 110 ml of culture media was removed, and increasing concentrations of FVIIa or FVIIa in combination with DEGR-FVIIa

10 were added to wells. A standard curve for FVIIa concentration was generated, ranging from 5 nM to 0.04 nM. To measure the inhibitory activity of DEGR-FVIIa on FVIIa activity, increasing concentrations of DEGR-FVIIa were added to test wells in the presence of a constant amount of FVIIa (5 nM). Both FVIIa and DEGR-FVIIa were diluted with HEPES buffer (10 mM HEPES, 137 mM NaCl, 4 mM KCl, 5 mM CaCl<sub>2</sub>, 11 mM glucose, 0.1% BSA)

15 and 10 ml of 10x stock solutions added to the cells. The cells were incubated with the test compounds for 2 hr at 37°C, then washed 3 times with HEPES buffer. Fifty microliters of a 200 nM solution of Factor X in Tris buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 2.7 mM KCl, 5 mM CaCl<sub>2</sub>, 0.1% BSA) was then added to each well. After 4 mins at room temp., 25 ml of 0.5 M EDTA was added to stop the Factor X to Xa conversion. Twenty-five microliters per

20 well of 0.8 mM S-2222, a factor Xa-specific chromogenic substrate, in Tris buffer was added and the absorbance at 405 nM read after 60 mins in a Thermomax microplate reader (Molecular Devices Corp., Menlo Park, CA).

The results, shown in Fig. 3, demonstrate a dose dependent increase in amidolytic activity for the FVIIa treated wells (open boxes). The increase in absorbance is a direct

25 measure of the level of Factor Xa generated in the wells and its subsequent cleavage of the chromogenic substrate. The addition of increasing amounts of DEGR-FVIIa with a constant amount of FVIIa (5 nM) showed a dose dependent decrease in amidolytic activity with increasing levels of DEGR-FVIIa (closed boxes). An equal molar ratio of DEGR-FVIIa to FVIIa was able to inhibit >90% of the chromogenic activity. Even at a 10-fold lower level of

30 DEGR-FVIIa, there was still a 40% inhibition in the generation of Factor Xa chromogenic activity. These results support the conclusion that DEGR-FVIIa is an extremely potent

antagonist of the activation of Factor X to Xa by FVIIa on the surface of intact cell monolayers of SMCs.

5 EXAMPLE XIV

Effect of DEGR-Factor VIIa on Vascular Thrombosis Formation and Vascular Lesion Formation in Baboons

Human DEGR-Factor VIIa was tested for the ability to inhibit tissue factor (TF) and  
10 activated Factor VII (FVIIa) mediation of vascular lesion formation (VLF) induced by mechanical vascular injury in nonhuman primates.

Beginning immediately prior to creating mechanical vascular injury in baboons, DEGR-Factor VIIa was infused intravenously for 7 days (5 animals) or 30 days (1 animal). Measurements were performed for vascular lesion formation on day 30. The results in 5  
15 treated animals were compared with the findings in 5 concurrent vehicle buffer-infused controls.

Baseline measurements were obtained on study animals for: a) platelet counts, neutrophil counts, monocyte counts and red cell counts; b) plasma fibrinogen level; c) activity levels of plasma coagulation factors VII, VIIa, X and V, together with the antigenic  
20 levels of FVII; and d) baseline plasma sample for anti-Factor VIIa antibody level.

Under halothane anesthesia and sterile operating conditions, animals labeled with autologous <sup>111</sup>In-platelets received intravenous infusions of DEGR-FVIIa using the tether system for continuous intravenous administration (initial bolus injection of 1 mg/kg followed by continuous intravenous infusion of 50 mg/kg/hr. The animals received surgical carotid  
25 endarterectomy, bilateral brachial artery or bilateral femoral artery Fogarty balloon catheter angioplasties.

The DEGR-FVIIa was administered for 7 or 30 days by continuous infusions via venous catheter using the tether system. Thirty days after surgery the animals were anesthetized with halothane and underwent *in situ* pressure-perfusion fixation with 4%  
30 paraformaldehyde containing 0.1% glutaraldehyde for 30 min. At that time, vascular segments (containing the sites previously injured) were harvested using procedures of Harker et al., Circulation 83:41-44 (1991) and Hanson et al., Hypertension 18:1170-1176

(1991). The specimens were post-fixed in vitro (4% paraformaldehyde containing 0.1% glutaraldehyde), cryopreserved and processed for morphometric analysis of lesion extent.

Eleven normal mature baboons (*Paio anubis*) were studied. Six animals received DEGR-FVIIa infusions (50 mg/kg/hr) and the remaining five were control animals that did not receive DEGR-FVIIa. The animals were dewormed and observed to be disease-free for three months prior to use. All procedures were approved by the Institutional Animal Care and Use Committee and were in compliance with procedures and methods outlined by NIH Guide for the Care and Use of Laboratory Animals, as well as the Animal Welfare Act and related institutional policies. Invasive procedures were carried out under halothane anesthesia after induction by ketamine (10 mg/kg intramuscularly) and valium (0.5 mg/kg intravenously). For subsequent short-term immobilization in performing experimental procedures postoperatively, ketamine hydrochloride (5-20 mg/kg intramuscularly) was used.

Carotid endarterectomy was performed through a midline neck incision using the technique of Hanson et al., Hypertension 18:1170-1-176 (1991) and Krupski et al., Circulation 84:1749-1757 (1991), incorporated herein by reference. Endarterectomy was used as a vascular injury model because of its clinical relevance, and because VLF induced by endarterectomy of normal arteries has been shown to be reproducible. In brief, the common carotid artery was dissected free of surrounding tissues from the clavicle proximally to the carotid bifurcation distally. The common carotid artery was cross-clamped using atraumatic vascular clamps placed at each end of the exposed vessel three minutes after a bolus injection of heparin sulfate (100 U/kg intravenously; Elkins-Simm Inc., Cherry Hill, NJ) and divided 1 cm proximal to the distal crossclamp. The proximal arterial segment was then everted over curved forceps. After maximal eversion was obtained, a pair of polypropylene stay sutures (7-0) was placed on either side proximally and a second pair placed distally in the lumen-exposed segment. The endarterectomy was then performed beginning 1 cm from the divided end of the everted vessel segment and continued for a measured distance of 1 cm. This procedure involves mechanical removal of the normal intima and a partial thickness of media using forceps and a surgical microscope (32X magnification). Following endarterectomy, the vessel was returned to its normal configuration, and an end-to-end anastomosis performed with 7-0 polypropylene suture and continuous technique under 2.5-fold magnification, and the wound closed in layers.

For morphometric analysis of VLF, sections embedded in paraffin and stained for connective tissue components (collagen, elastin) and with hematoxylin-eosin, were evaluated using a Zeiss Photoscope coupled with image analysis system (Thomas Optical Measurement Systems, Columbus, GA) consisting of high resolution (580 lines) CCD  
5 microscope camera coupled to a high resolution (700 lines) monitor, an IBM 386 chip, 80 MB computer with high resolution graphics digitablet for image acquisition and storage. Quantitative image analysis was performed using a morphometric software driver (Optimas, Bioscan, Inc., Edmonds, WA). Arterial cross-sections were analyzed with respect to the total area of neointimal proliferative lesion and corresponding area of arterial media. For  
10 statistical analysis, comparisons between groups were made using the Student's t test (two tailed) for paired and unpaired data.

The results showed that the intimal area was significantly decreased in the animals treated with DEGR-Factor VIIa for seven days and studied at 30 days as compared to control animals who had undergone the same vascular injury but who did not obtain any  
15 DEGR-Factor VIIa (Fig. 4). A similar result was found in the animal treated with DEGR-Factor VIIa for 30 days and examined at 30 days.

Preliminary studies with a balloon angiographic brachial artery model suggested no measurable benefit of DEGR-Factor VIIa therapy. This model, however, has not been shown in baboons to be a prothrombotic model in which tissue factor plays a key role.

20 Studies with the femoral artery balloon injury in the baboon did show a statistically significant benefit from DEGR-Factor VIIa as compared to controls, as shown in Fig. 5.

#### EXAMPLE XV

##### Effect of DEGR-Factor VIIa on tPA-Induced Thrombolysis

25 Ongoing coronary thrombus formation during acute myocardial infarction is primarily mediated by tissue factor (TF) in complex with Factor VIIa through the extrinsic coagulation pathway. The effect of adjunctive coagulation cascade inhibition at different points in the extrinsic pathway on the efficiency of tissue plasminogen activator (TPA) thrombolysis was determined.

30 Thirty-six dogs with electrically-induced coronary thrombus undergoing thrombolysis with tPA (1 mg/kg over 20 min) were given 1 of 4 adjunctive treatments: 9 received tick anticoagulant peptide (TAP), a selective factor Xa inhibitor, at 30 mg/kg/min for 90 min. TF-



Factor VIIa complex was inhibited by recombinant tissue factor pathway inhibitor (TFPI) (100-150 mg/kg/min for 90 min) in 9 dogs, and by DEGR VIIa (1-2 mg/kg bolus) as a competitive antagonist of activated Factor VIIa in 9 dogs. Nine dogs received a saline control. Dogs were observed for 120 minutes after thrombolysis for reocclusion. The effects of these agents on the efficiency of thrombolysis are shown below in Table 18 (data as mean  $\pm$  SD).

Table 18.

	Saline	DEGR-FVIIa	TFPI	TAP
Time to reflow (min)	32 $\pm$ 13	20 $\pm$ 7*	21 $\pm$ 6*	18 $\pm$ 10*
Reflow duration (min)	62 $\pm$ 45	70 $\pm$ 48	91 $\pm$ 35*	120
Cycle flow variations	70%	89%	56%	0%
Reocclusion	70%	78%	67%	0%

\*Value different from saline control at a level 0.05 of significance.

These data indicate that extrinsic pathway inhibition by either Factor Xa or TF-Factor VIIa blockade by DEGF VIIa or TFPI accelerated tPA-induced thrombolysis. Selective inhibition of Factor Xa more efficiently maintained arterial patency following successful reperfusion.

#### EXAMPLE XVI

##### Modified Factor VIIa Inhibits Intravascular Thrombus Formation Without Affecting Systemic Coagulation

To determine whether inhibition of Factor VII binding to TF would result in antithrombotic effects, cycle flow variations (CFVs) due to recurrent thrombus formation were initiated by placing an external constrictor around endothelially-injured rabbit carotid arteries (Fotts' model). Carotid blood flow was measured continuously by a Doppler flow

probe placed proximally to the constrictor. After positioning the constrictor around the artery, CFVs developed with a mean frequency of  $11 \pm 2$  cycles/hr in 6 of 6 rabbits, whereas carotid blood flow velocity averaged  $5 \pm 2\%$  of baseline values at the nadir of CFVs. After CFVs were observed for 30 min, the animals received an infusion of human recombinant active site-  
5 blocked (Phe-Phe-Arg chloromethylketone) Factor VIIa (FVIIai) (0.1 mg/kg/min for 10 min). The Factor VIIai completely abolished CFVs in 6 of 6 animals (CFV frequency = 0 cycles/hr;  $p < 0.05$ ; carotid blood flow velocity = 106.9% of the baseline values;  $p = \text{NS}$  vs. baseline). Thirty minutes following inhibition of CFVs, human recombinant FVIIa was infused at the doses of 0.1 mg/kg/min for 10 min. Infusion of the Factor VIIa restored CFVs in all animals,  
10 thus indicating that Factor VIIa binding to TF was competitive. Prothrombin times, activated partial thromboplastin times, and *ex vivo* platelet aggregation in response to ADP and thrombin were not different after FVIIai infusion as compared to baseline values. Thus, FVII-VIIa plays an important role in initiating thrombus formation *in vivo*. Administration of Factor VIIai exerts potent antithrombotic effects in this model without affecting systemic  
15 coagulation.

#### EXAMPLE XVII

##### Inhibition of Microarterial Thrombosis by Topical Administration of Modified Factor VIIa

20 In vascular surgery, microvascular reconstructive surgery, or replantation surgery the most common cause of failure is thrombosis at the anastomotic sites. The risk of occlusive thrombus formation is highly increased when vessels have been subjected to trauma, exhibit pathological changes, or when interpositional vein grafts are used. Therefore, antithrombotic intervention in context with surgery is frequently used. The  
25 substances which are currently available and used on this indication are administered parenterally (heparin, dextran) or orally (ASA) and are all associated with hemorrhagic side-effects. Moreover, heparin and especially ASA are only partially effective in preventing (arterial) thrombus formation. Based on these drawbacks, a need exists to prevent thrombosis in vascular surgery using an agent that binds to and is effective in anastomotic  
30 regions and sites of vascular trauma by means of a substance that can be delivered locally, thereby avoiding undesirable systemic side-effects. In this Example, topical administration of active-site inactivated Factor VIIa at arterial trauma sites was used to produce an

antithrombotic effect without inducing a tendency for increased bleeding or other hemostatic defect.

5

#### Methods:

Swedish loop rabbits of either sex weighing ~3.5 kg were fed a standard pellet diet and given water ad libitum. They were observed to be disease-free at the laboratory for at least one week before use.

A marginal ear vein was cannulated and anaesthesia induced with sodium pentobarbital, 18 mg/kg, and maintained by repeated injections.

Skin flaps were raised on both ears, and 3 cm long segments of the central arteries (outer diameter ~1mm) prepared. All branches were ligated with 10-0 sutures and cut. The operative field was superfused with isotonic saline and covered with thin plastic films. To keep blood-flow high and constant and counteract vasospasm, the animals were placed on heat pads and kept slightly hyperthermic at a body temperature of ~39.5° C (normal body temperature ~38.5° C) and three drops of lidocaine 10 mg/ml were applied topically to the vessels after manipulating them (after reperfusion and after testing patency at 30 minutes after reperfusion).

Vessels on both sides were simultaneously placed in double microvascular clamps (S&T 2V, S&T Marketing Ltd., Neuhausen, Switzerland), thereby isolating 7 mm artery segments between the clamps. Longitudinal arteriotomies (7 mm) were performed, whereafter the clamps were approximated, the vessels repositioned and the vascular lumina everted and flattened, exposing deep layers of the tunica media. The arteriotomies were closed with continuous 10-0 monofilament nylon sutures (Ethilon 10-0 BV-75-3, Ethicon Ltd., Edinburgh, U.K.). All surgical procedures were carried out by one surgeon using a high-quality operating microscope (Wild M-650, Leica-Heerbrugg, Heerbrugg, Switzerland).

Vessels were simultaneously reperfused by opening the vascular clamps. They were quickly covered by saline-soaked gauze pads, and inspected once a minute. The time until complete cessation of arteriotomy bleeding was recorded.

At 30 and 120 min. after reperfusion, vessel patency was assessed using a standard microsurgical empty/refill test: Vessels were gently occluded distal to the trauma area with a pair of microforceps, and emptied downstream with another pair of forceps. After release of the first pair of forceps, vessel refilling was assessed and vessels classified as patent or occluded. Occluded vessels showed no refilling, while patent vessels showed rapid or slow refilling, the latter being referred to as "reduced patency". After the final patency test, vessels were excised and opened longitudinally, whereafter the thrombotic material was removed and weighed.

10 Compounds:

Recombinant chemically-inactivated human factor VIIa (VIIai) at a concentration of 3.1 mg/ml or vehicle were stored as 200 ml aliquots in coded vials.

Experimental protocol:

15 Twenty rabbits were treated as follows in a blind random fashion, each rabbit serving as its own control: After performing the deep arterial trauma as described in the method section above, the exposed trauma site on one ear was superfused during 5 minutes with VIIai solution (a total of 0.5 mg) and on the other ear with vehicle. The superfused trauma fields were allowed to incubate with the solutions during an additional 5 minute  
20 period, whereafter all surplus of solution was flushed away with isotonic saline. The arteriotomies were then closed and the vessels reperused.

Statistical methods:

25 Patency results were compared using the sign test, and thrombus weights and arteriotomy bleeding data with the Wilcoxon test. Two-sided p values were presented.

Results:

The administration of VIIai gave a distinct antithrombotic effect as measured by patency rates. In the VIIai group the vessel patency was 85% at 30 minutes and 75% at 120  
30 after reperfusion. Corresponding values in the vehicle group were 40% and 30% respectively. The difference is statistically significant ( $p=0.008$  and  $p=0.004$  respectively). Median thrombus weights were 0.3 mg in the VIIai group and 0.5 mg in the vehicle group,

although this difference was not significant ( $p=0.19$ ). Median arteriotomy bleeding times were 1.5 minutes in the VIIai group and 2 minutes in the vehicle group. The groups are statistically indistinguishable ( $p=1$ ).

- 5 This Example demonstrates that topical administration of VIIai at arterial trauma sites produces an antithrombotic effect without inducing an increased bleeding tendency. This presents a highly attractive mode of treatment for preventing thrombotic complications due to surgery, microsurgery on blood vessels, angioplasty or other trauma.

#### 10 EXAMPLE XVIII

##### Local Application of FVIIai Reduces Thrombus-Weight and Improves Patency

This Example demonstrates that local application of chemically inactivated FVIIa (FVIIai) reduces thrombus-weight and improves vascular patency.

- 20 Twenty anesthetized rabbits were used in this Example. The jugular veins were mobilized and a 10 mm segment was isolated between clamps. The thrombus was introduced by a combination of chemical (aetoxysclerol) destruction of the endothelium of the isolated segment and a semi-restricting ligature placed caudally of the segment. In a blinded randomized fashion one side was treated with 0.5 mg chemically inactivated FVIIa (FVIIai) and the other with the buffer. The test substance was injected to the isolated segment and incubated for 10 min. after the chemical destruction. 30 and 120 min. later patency was controlled with an empty/refill test. Possible thrombus was weighed after sacrifice.

	median thromb. weight	range of thromb. weights	p-value	30 min. patency	p-value	120 min. patency	p-value
FVIIai	0.85 mg	0-22.3 mg	0.035	90 %	0.0070	85 %	0.070
buffer	9.3 mg	0-26.8 mg		55 %		50 %	

The results showed that local application of inactivated FVIIa significantly reduced thrombus-weight and improved patency in the venous thrombosis model.

5

#### EXAMPLE XIX

##### Application of FVIIai Reduces the Area of Risk and improves reperfusion

##### Methods

##### 10 *Experimental Preparation*

Twenty-eight New Zealand white rabbits of both sexes (3.2-3.8 kg) were studied. Briefly, the animals were anesthetized with a mixture of ketamine (35 mg/kg) and xylazine (5mg/kg) administered intramuscularly, intubated and ventilated with a constant volume respirator (harvard Apparatus Co., Cambridge, MA). Polyethylene catheters were placed in the aorta  
15 through the left carotid artery and into a jugular vein for monitoring arterial pressure and administration of drugs, respectively. A thoracotomy was performed through the fifth left intercostal space and the pericardium opened. A polyethylene catheter was placed into the left atrial appendage for later injections of coloured microspheres. The large marginal branch of the circumflex coronary artery was temporarily occluded approximately 0.3 cm from its  
20 origin with a surgical suture snare. Coronary artery occlusion was maintained for 30 min, at which time the ligature was released and reperfusion allowed for additional 5.5 hours. Systemic arterial pressure (Statham P23 DB pressure transducer) was recorded continuously during the experiment (Gould Instruments).

##### *Experimental Protocol*

25 At the moment of reperfusion, the animals were randomly assigned to one of the following treatment groups: A control group received a 5-ml bolus of saline into the left atrium; a group treated with human recombinant, active site-blocked factor VIIa (FVIIai, Novo Nordisk A/S, Gentofte, Denmark, 1 mg/kg bolus into the left atrium); a group treated with human recombinant activated factor VIIa (FVIIai, Novo Nordisk A/S, Gentofte, Denmark, 1 mg/kg  
30 bolus into the left atrium).

##### *Assessment of Area of Risk, Infarct Size, and No-Reflow phenomenon*

To estimate the distribution of tissue perfusion at the end of the experiment ("no-reflow" phenomenon, NR) the animals received an injection of a 6% solution of thioflavin S (1 mg/kg) via the left atrial catheter. To permit the assessment of the area of risk of infarcting, the coronary artery was reoccluded immediately after injecting thioflavin, and a solution of monastral blue (E.I.DuPont; 1 mg/kg) was injected through the left atrial catheter. The heart was thereafter immediately excised and the left ventricle was dissected free from all other structures and weighed. The left ventricle was frozen at -70 °C for 30 min and cut into 8 to 10 slices parallel to the atrioventricular groove. Contours of the normally perfused myocardium as well as of the area of risk, according to the distribution of monastral blue, were traced onto a transparent plastic sheet. Myocardial slices were then observed under ultraviolet light and the normally perfused myocardium (fluorescent) was then easily differentiated and separated from the ischemic myocardium (nonfluorescent) according to the thioflavin distribution. These areas were also traced onto transparent plastic sheets. The slices were next incubated in a 2% solution of triphenyltetrazolium chloride (TTC, Sigma Chemicals) for 10 min at 37 °C, to visualize the area of necrosis. Again, a transparent plastic sheet was used to trace the contours of the normal myocardium (TTC-positive), and of the infarcted portion (TTC-negative).

The following variables were calculated: 1) the area of risk of infarction, as a percent of the left ventricle, assessed at the end of the reperfusion period (monastral blue distribution) (AR); 2) infarct size (IS), as a percent of the area of risk that actually evolved to necrosis by the TTC staining criterion; 3) the percentage of the area of risk that did not receive blood flow at the end of the reperfusion period (no-reflow phenomenon, NR).

#### *Regional Myocardial Blood Flow measurements.*

Regional myocardial blood flow (RMBF) was measured in all rabbits in each treatment group. Differentially coloured plastic microspheres (Blue, Red, and Yellow, triton Technology, san Diego, CA) were used to measure RMBF 20 min after occlusion and 10 min and 5 hrs after reperfusion.

Microspheres were  $15 \pm 1 \mu$  in size and suspended in 10% dextran solution with 0.01% Tween 80. To ensure adequate dispersion, the microspheres were sonicated in an ultrasonic bath for 5 min immediately before use. Approximately 500,000 microspheres were injected (0.5 - 1.0 ml total volume) into the left atrial catheter. One minute before microsphere injection, reference arterial blood flow withdrawal was begun and continued for 1 min after

the injection. Tissue samples (100 to 300 mg) from the center of the ischemic areas and from nonischemic regions were taken according to TTC staining. The microspheres were then recovered from tissue by digestion in a 4 M KOH solution at 72 °C for 3 hrs and from reference blood samples by digestion in 16 M KOH at room temperature for 3 hrs and subsequent microfiltration, according to the instructions provided by the manufacturer. The dyes were then recovered from the spheres within a known volume of a solvent (dimethylformamide) and their concentrations determined by spectrophotometry at optimal wave lengths for each dye according to the manufacturer's instructions. The composite spectrum of each dye solution was resolved into the spectra of the single constituents by a matrix inversion technique. Blood flow to each myocardial sample was calculated by the formula:  $RMBF = Fr \times Am/Ar$ , where RMBF = myocardial blood flow in ml/min, AM = absorbance in myocardial sample, and AR = absorbance in reference blood sample. Myocardial blood flow was divided by the sample wet weight and expressed as ml/min/g.

#### *Coagulation Studies*

To determine the effects of FVIIa and FVIIa administration on systemic coagulation, prothrombin time (PT) and activated partial thromboplastin time (aPTT) were measured at baseline and 30 minutes after drug administration. Blood samples (4.5 ml) were collected in 0.5 ml sodium citrate (3.8%) and centrifuged at 2000 g for 10 min at 4 °C to separate the plasma. PT and aPTT were measured in duplicate within 2 hrs from blood collection.

#### *Statistical Analyses*

Results are expressed as mean  $\pm$  SD of the mean. Analysis of variance was used for multiple comparisons among groups. Differences for individual groups were tested with Student's t-test for unpaired observations with Bonferroni's correction. For comparisons of hemodynamic variables, as well as regional myocardial blood flow among groups, a two-way analysis of variance with a design for repeated measures was used.

### Results

Twenty-eight rabbits underwent the surgical procedure; two animals died during coronary occlusion for ventricular fibrillation before treatment group allocation and two additional rabbits died during reperfusion (one in the control and one in the FVIIa-treated group). These animals were excluded from subsequent statistical analysis. Therefore, eight animals in each treatment group were included in the study.



*Hemodynamic Measurements:*

In all treatment groups, coronary occlusion induced a slight decrease in heart rate and mean arterial pressure. No differences were found among the three groups in heart rates and mean arterial pressures during the course of the experimental periods (Table I).

5                   *Assessment of area of Risk, Infarct Size, and the No-Reflow Phenomenon:*

Coronary occlusion produced an area of risk of infarction assessed by injection of monastral blue at the end of the experiment which was similar in the three treatment groups ( $31.6 \pm 6.3$ ,  $28.2 \pm 4.1$ , and  $29.2 \pm 5.3\%$  of the left ventricle in control, FVIIai, and FVIIa-treated animals, respectively,  $p=NS$ ).

10                   After 30 min of coronary occlusion and 5.5 hrs of reperfusion, the amount of the area at risk that evolved toward necrosis averaged  $59.8 \pm 12.8\%$  in the control group (Fig. 7). Administration of FVIIai significantly reduced infarct size to  $28.1 \pm 11.3\%$  of the area at risk  $p<0.01$  by ANOVA, Fig. 1), while FVIIa administration was associated with a significant increase in infarct size to  $80.1 \pm 13.1\%$  of the area of risk ( $p<0.01$  vs. controls and FVIIai-  
15                   treated rabbits, Fig. 7).

In control rabbits,  $24.4 \pm 2.7\%$  of the area of risk showed a perfusion defect, as determined by thioflavine S distribution at the end of the experiment (no-reflow phenomenon). The extent of this area of no-reflow was significantly reduced by FVIIai and significantly increased by FVIIa to  $11.1 \pm 6.1$  and  $61.9 \pm 13.8\%$  of the area of risk, respectively ( $p<0.01$ ,  
20                   Fig. 8).

Previous studies have established that the amount of the myocardial tissue that shows a perfusion defect during post-ischemic reperfusion is related to various parameters; the most important are the extent of the area of risk, the magnitude of infarct size, and the amount of residual collateral flow during occlusion. Controlling for these variables allows for  
25                   more precise assessment of the effects of interventions on the no-reflow phenomenon. In the present study, when the no-reflow area in control rabbits was correlated to these parameters, a close relationship was observed, which fits a multiple linear regression equation:  
$$NR (\% \text{ of the left ventricle [LV]}) = -14.62 + 0.75(AR) + .07(IS) + 3.69(RMBF); r^2 = 0.98; F \text{ test} = 109.3, (0.37)(0.3)(3.69),$$
  
where NR is no-reflow area, AR is the area at risk, and RMBF  
30                   is collateral blood flow (in ml/min/g). The number in parentheses indicate the standard errors of the coefficients. With an  $r^2$  value of 0.98, this model accounted for >95% of the variation in the no-reflow area that was observed in control animals in this study.

The equation coefficients that were obtained from the data of control rabbits in the multiple regression equation were then used to calculate the expected no-reflow areas for individual animals within each intervention group (Fig. 9). The actually observed no-reflow areas in rabbits receiving FVIIai were significantly smaller than the expected ones calculated applying the equation obtained from the multiple regression analysis. Indeed, in FVIIai-treated rabbits, for any given expected area of no-reflow, the actual observed value was smaller, such that all animals in this group distributed below the regression line obtained for control animals (Fig. 9). On the contrary, FVIIa-treated rabbits showed just the opposite, i.e., for any given expected area of no-reflow, the actual observed value was significantly bigger, as all animals distributed above the regression line of control animals (Fig. 9). Taken together, these data indicate that the reduction in the no-reflow phenomenon observed in FVIIai treated animals is not entirely accounted for by the reduction in infarct size, and suggest that activation of the extrinsic coagulation cascade during post-ischemic reperfusion contribute to the occurrence of the no-reflow phenomenon.

15        *Regional Myocardial Blood Flow Measurements:*

In control animals RMBF to the non-ischemic myocardium averaged 1.20 ml/min/g of tissue throughout the study (data not shown). In the same group of animals, RMBF to the ischemic myocardium was  $0.08 \pm 0.02$ ,  $1.43 \pm 0.28$ , and  $0.98 \pm 0.19$  ml/min/g of tissue 20 min after coronary occlusion and 10 min and 5 hrs after perfusion, respectively. The various drug treatment used in the present study did not significantly change RMBF during the experimental period both in the normal and in the ischemic myocardium as compared to control animals (Fig. 10).

*Coagulation Studies:*

To study possible systemic effects of FVIIai, which may predispose to an increased risk of bleeding, PTs and aPTTs were measured in blood samples collected at 30 minutes of CFVs, and after FVIIai and FVIIa administration. At the end of the 30 min CFVs period, PTs and aPTTs averaged  $8.2 \pm 0.6$  seconds and  $25 \pm 3$  seconds, respectively. A slight increase in PTs to  $10.1 \pm 0.6$  seconds was observed after FVIIai administration (Fig. 11). This increase, however, did not reach statistical significance ( $p=0.09$  by ANOVA and student's t-test with the Bonferroni's correction). APTT did not change significantly after FVIIai administration (Fig. 10). FVIIa administration resulted in a significant shortening in both PTs and aPTTs only with respect to the values obtained after FVIIai administration (Fig. 11).

Table 1:

Hemodynamic variables during coronary artery occlusion-reperfusion

Heart Rate (b/min)					
Time after occlusion	Control	SQ29548	Dazoxiben	R68070	ASA
0	175 ± 5	170 ± 4	178 ± 6	169 ± 5	1
30 min	169 ± 4	165 ± 5	173 ± 5	164 ± 6	1
1 hr	169 ± 6	167 ± 4	171 ± 5	166 ± 5	1
2 hr	173 ± 5	172 ± 5	175 ± 6	170 ± 5	1
3 hr	170 ± 4	168 ± 5	177 ± 5	168 ± 6	1
4 hr	175 ± 5	168 ± 6	174 ± 5	169 ± 5	1
5 hr	175 ± 5	172 ± 2	173 ± 5	172 ± 6	1
6 hr	168 ± 5	170 ± 4	174 ± 5	168 ± 5	1

Mean arterial pressure (mm Hg)					
Time after occlusion	Control	SQ29548	Dazoxiben	R68070	ASA+R68070
0	75 ± 4	78 ± 3	78 ± 4	73 ± 3	7
30 min	69 ± 4	65 ± 4	71 ± 3	67 ± 4	6
1 hr	69 ± 3	67 ± 4	71 ± 4	69 ± 4	6
2 hr	73 ± 4	75 ± 4	75 ± 5	72 ± 4	7
3 hr	74 ± 4	75 ± 3	77 ± 5	75 ± 3	7
4 hr	75 ± 5	78 ± 4	76 ± 4	73 ± 4	7
5 hr	73 ± 4	74 ± 5	78 ± 5	72 ± 4	7
6 hr	73 ± 5	76 ± 4	74 ± 5	73 ± 5	7

Table II

Regional myocardial blood flow (ml/min/g of tissue) during coronary occlusion and reperfusion

	Control	FVIIai	FVIIa
Normal myocardium			
20 min CAO	1.19 ± 0.22	1.03 ± 0.19	1.27 ± 0.24
10 min REP	1.22 ± 0.15	1.10 ± 0.17	1.19 ± 0.20
2 hrs REP	1.17 ± 0.18	1.07 ± 0.16	1.22 ± 0.19
Ischemic myocardium			
20 min CAO	0.09 ± 0.05	0.08 ± 0.05	0.08 ± 0.04
10 min REP	1.53 ± 0.12	1.65 ± 0.18	1.24 ± 0.14
2 hrs REP	0.89 ± 0.14	1.23 ± 0.15	0.72 ± 0.13

5

CAO = Coronary Artery Occlusion; REP = Reperfusion

## EXAMPLE XIX

Application of FVIIai Reduces Infarct size and Area at Risk of Infarction

Tissue factor exposure occurs during reperfusion of post-ischemic hearts within the coronary vasculature, leading to a decrease in coronary blood flow.

To assess whether tissue factor exposure might contribute to myocardial injury via activation of the coagulation and reduction in coronary blood flow during post-ischemic reperfusion, NZW rabbits underwent 30 min coronary occlusion followed by 5.5 hrs of reperfusion. At reperfusion, the animals randomly received: saline (n=8); human recombinant, active site-blocked factor VIIa (FVIIai, 100 µg/kg/min for 10 minutes into the left atrium, n=8) or human recombinant activated factor VIIa (FVIIa, 100 µg/kg/min into the left atrium, n=8). Regional myocardial blood flow (RMBF) was measured using coloured microspheres at 20 min of ischemia, and 10 min and 2 hrs following reperfusion. The area at risk of infarction (AR), infarct size (IS), and the no-reflow area (NR) were determined at the end of the experiment.

riment by monastral blue and thioflavin distribution and by TTC staining. FVIIai resulted in a significant reduction in both IS and NR with respect to controls ( $28.1 \pm 11.3\%$  and  $11.1 \pm 6.1\%$  of AR vs.  $59.8 \pm 12.8\%$  and  $24.4 \pm 8.2\%$  of AR, respectively,  $p < 0.01$ ), while FVIIa resulted in a significant increase in both IS and NR to  $80.1 \pm 13.1\%$  and  $61.9 \pm 13.8\%$  of AR, respectively,  $p < 0.01$  vs. Controls. No differences in blood pressure, heart rate, AR, and RMBF at 20 min of ischemia were observed among groups. RMBF was significantly higher at 2 hrs of reperfusion in FVIIai-treated animals, while it was lower in FVIIa-treated rabbits. Thus, TF-mediated activation of the coagulation importantly contributes to the occurrence of myocardial injury during post-ischemic reperfusion.

10

	FVIIai (AR)	Control (AR)	FVIIa (AR)
IS	$28.1 \pm 11.3$	$59.8 \pm 12.8$	$80.1 \pm 13.1$
NR	$11.1 \pm 6.1$	$24.4 \pm 8.2$	$61.9 \pm 13.8$

AR = The area at risk of infarction; IS = infarct size (IS); NR = the no-reflow area

15            Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

5

(i) APPLICANT: ZymoGenetics, and  
Novo Nordisk A/S

10

(ii) TITLE OF INVENTION: Modified Factor VII

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:

15

(A) ADDRESSEE: Novo Nordisk A/S, Corporate Patents  
(B) STREET: Novo Allé  
(C) CITY: DK-2880 Bagsvaerd  
(D) COUNTRY: Denmark

20

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.24

25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

30

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 08/475,845  
(B) FILING DATE: 07-JUN-1995  
(C) CLASSIFICATION:

35

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 2422 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

10

## (iii) HYPOTHETICAL: N

## (iv) ANTI-SENSE: N

15

## (ix) FEATURE:

- (A) NAME/KEY: CDS  
(B) LOCATION: 28..1420  
(D) OTHER INFORMATION: /codon\_start= 28  
/product= "Factor VII"

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Leu Arg Leu Leu Cys Leu Leu Leu Gly Leu Gln Gly Cys Leu Ala Ala  
-30 -25 -20

30 GTC TTC GTA ACC CAG GAG GAA GCC CAC GGC GTC CTG CAC CGG CGC CGG 151  
Val Phe Val Thr Gln Glu Glu Ala His Gly Val Leu His Arg Arg Arg  
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	Gln Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg	
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25	GGC GGC TGT GAG CAG TAC TGC AGT GAC CAC ACG GGC ACC AAG CGC TCC	487
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	Gln Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala	
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	340 345 350	
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ACTCAAAGAG ACTCCAAGAT TCAAAGAGAC TAATAGAGAC ACAGAGATGG AATAGAAAAG 1636

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20 CGCACACATC AGTGCACACG GATGCACAGA GATATGCACA CACCGATGTG CGCACACACA 2056

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AATTCAGAAG CATCACCATG CATGGTGGCG AATGCCCCCA AACTCTCCCC CAAATGTATT 2296

30 TCTCCCTTCG CTGGGTGCCG GGCTGCACAG ACTATTCCCC ACCTGCTTCC CAGCTTCACA 2356

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35 AAAAAA 2422

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 444 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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15                    20                    25

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Gly Ser Cys Lys Asp Gln Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro  
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Ala Phe Glu Gly Arg Asn Cys Glu Thr His Lys Asp Asp Gln Leu Ile  
75                    80                    85                    90

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## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 21 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

10

## (iii) HYPOTHETICAL: N

## (iv) ANTI-SENSE: N

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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## (2) INFORMATION FOR SEQ ID NO:4:

## 25 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

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## (ii) MOLECULE TYPE: cDNA

## (iii) HYPOTHETICAL: N

## 35 (iv) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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## CLAIMS

1. A method for inhibiting thrombus formation in a patient comprising administering topically to a vascular site susceptible to thrombus formation in the patient a therapeutically effective dose of a composition comprising Factor VII having at least one modification in its catalytic center, which modification substantially inhibits the ability of the modified Factor VII to activate plasma Factor X or IX.  
5
2. A method according to claim 1, wherein the modification comprises reaction of the Factor VII with a serine protease inhibitor.  
10
3. A method according to claim 2, wherein the protease inhibitor is an organophosphor compound, a sulfanyl fluoride, a peptide halomethyl ketone, or an azapeptide.
- 15 4. A method according to claim 3, wherein the protease inhibitor is a peptide halomethyl ketone selected from Dansyl-Phe-Pro-Arg chloromethyl ketone, Dansyl-Glu-Gly-Arg chloromethyl ketone, Dansyl-Phe-Phe-Arg chloromethyl ketone and Phe-Phe-Arg chloromethyl ketone.
- 20 5. The method according to any of claims 1 to 4, wherein the site of thrombus formation is associated with surgery, microsurgery, angioplasty or trauma.
6. A method for maintaining or improving vascular patency in a patient comprising administering locally to a vascular site susceptible to decreased patency a therapeutically effective dose of a composition comprising Factor VII having at least one modification in its catalytic center, which modification substantially inhibits the ability of the modified Factor VII to activate plasma Factor X or IX.  
25
7. A method according to claim 6, wherein the modification comprises reaction of the Factor VII with a serine protease inhibitor.  
30

8. A method according to claim 7, wherein the protease inhibitor is an organophosphor compound, a sulfanyl fluoride, a peptide halomethyl ketone, or an azapeptide.
9. A method according to claim 8, wherein the protease inhibitor is a peptide halomethyl ketone selected from Dansyl-Phe-Pro-Arg chloromethyl ketone, Dansyl-Glu-Gly-Arg chloromethyl ketone, Dansyl-Phe-Phe-Arg chloromethyl ketone and Phe-Phe-Arg chloromethylketone.
10. The method according to any of claims 6 to 9, wherein the site of decreased patency is associated with surgery, microsurgery, angioplasty or trauma.
11. Use of Factor VII having at least one modification in its catalytic center, which modification substantially inhibits the ability of the modified Factor VII to activate plasma Factor X or IX, for the manufacture of a composition for prevention or minimizing of myocardial injury associated with post-ischemic reperfusion.
12. Use according to claim 16, wherein the modification comprises reaction of the Factor VII with a serine protease inhibitor.
13. Use according to claim 17, wherein the protease inhibitor is an organophosphor compound, a sulfanyl fluoride, a peptide halomethyl ketone, or an azapeptide.
14. Use according to claim 18, wherein the protease inhibitor is a peptide halomethyl ketone selected from Dansyl-Phe-Pro-Arg chloromethyl ketone, Dansyl-Glu-Gly-Arg chloromethyl ketone, Dansyl-Phe-Phe-Arg chloromethyl ketone and Phe-Phe-Arg chloromethylketone.
15. Use according to any of claims 16 to 19, wherein the myocardial injury is myocardial necrosis.
16. A method for preventing or minimizing myocardial injury associated with post-ischemic reperfusion in an individual, comprising administering to the individual a composition which comprises a pharmacologically acceptable Factor VII having at least one modifica-

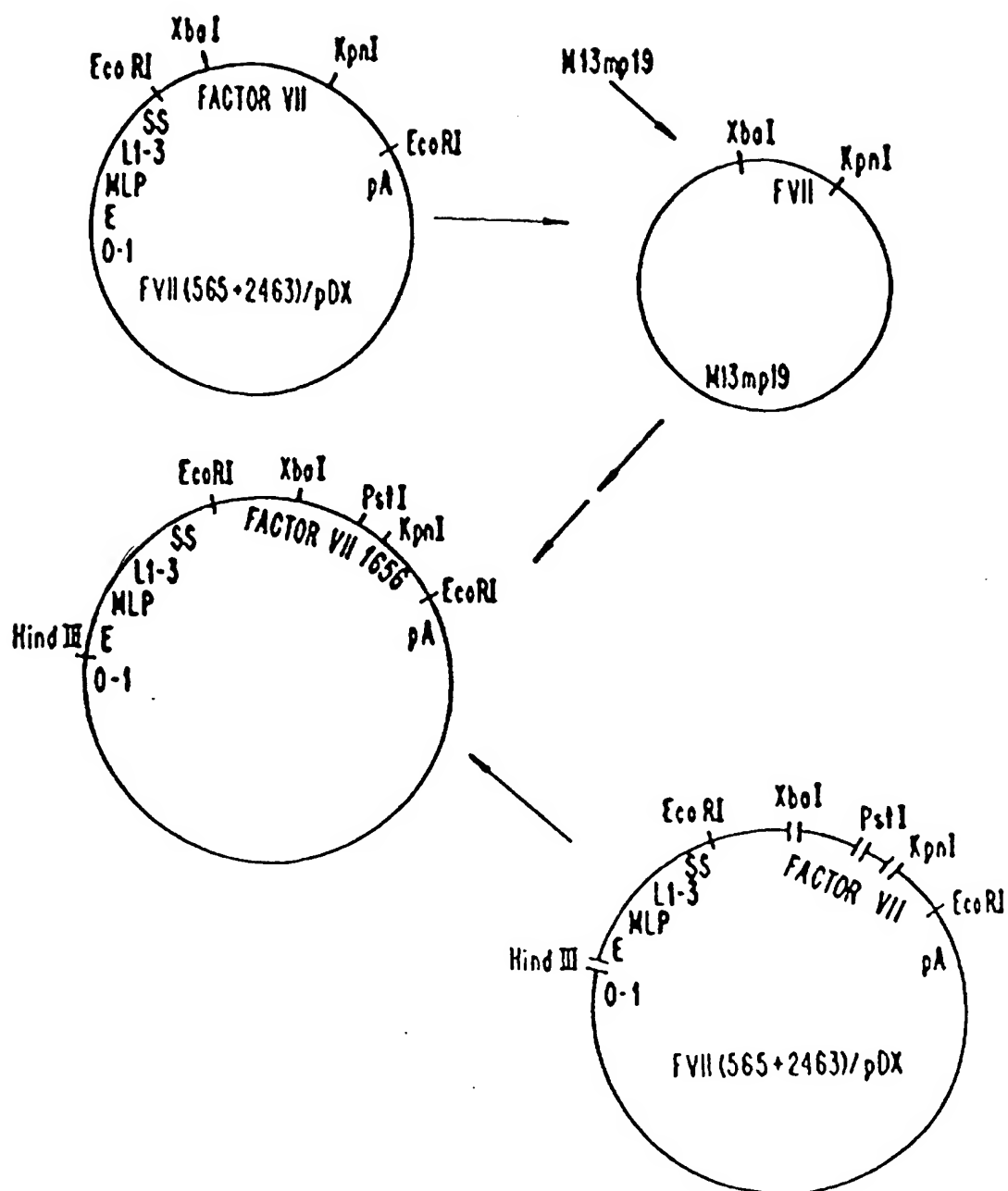
tion in its catalytic center, which modification substantially inhibits the ability of the modified Factor VII to activate plasma Factor X or IX.

17. The method according to claim 21, wherein the modification comprises reaction of the  
5 Factor VII with a serine protease inhibitor.
18. The method according to claim 22, wherein the protease inhibitor is an organophosphor compound, a sulfanyl fluoride, a peptide halomethyl ketone, or an azapeptide.
- 10 19. The method according to claim 23, wherein the protease inhibitor is a peptide halomethyl ketone selected from Dansyl-Phe-Pro-Arg chloromethyl ketone, Dansyl-Glu-Gly-Arg chloromethyl ketone, Dansyl-Phe-Phe-Arg chloromethyl ketone and Phe-Phe-Arg chloromethylketone.
- 15 20. The method according to any of claims 21 to 24, wherein the myocardial injury is myocardial necrosis.
21. Use of Factor VII having at least one modification in its catalytic center, which modification substantially inhibits the ability of the modified Factor VII to activate plasma Factor X  
20 or IX, for the manufacture of a composition for improving regional myocardial blood flow during post-ischemic reperfusion.
22. Use according to claim 26, wherein the modification comprises reaction of the Factor VII with a serine protease inhibitor.
- 25 23. Use according to claim 27, wherein the protease inhibitor is an organophosphor compound, a sulfanyl fluoride, a peptide halomethyl ketone, or an azapeptide.
24. Use according to claim 28, wherein the protease inhibitor is a peptide halomethyl ketone  
30 selected from Dansyl-Phe-Pro-Arg chloromethyl ketone, Dansyl-Glu-Gly-Arg chloromethyl ketone, Dansyl-Phe-Phe-Arg chloromethyl ketone and Phe-Phe-Arg chloromethylketone.

25. A method for improving regional myocardial blood flow during post-ischemic reperfusion in an individual, comprising administering to the individual a composition which comprises a pharmacologically acceptable Factor VII having at least one modification in its catalytic center, which modification substantially inhibits the ability of the modified Factor VII to activate plasma Factor X or IX.
26. A method according to claim 30, wherein the modification comprises reaction of the Factor VII with a serine protease inhibitor.
27. A method according to claim 31, wherein the protease inhibitor is an organophosphor compound, a sulfanyl fluoride, a peptide halomethyl ketone, or an azapeptide.
28. A method according to claim 32, wherein the protease inhibitor is a peptide halomethyl ketone selected from Dansyl-Phe-Pro-Arg chloromethyl ketone, Dansyl-Glu-Gly-Arg chloromethyl ketone, Dansyl-Phe-Phe-Arg chloromethyl ketone and Phe-Phe-Arg chloromethylketone.

1/11

Fig. 1



2/11

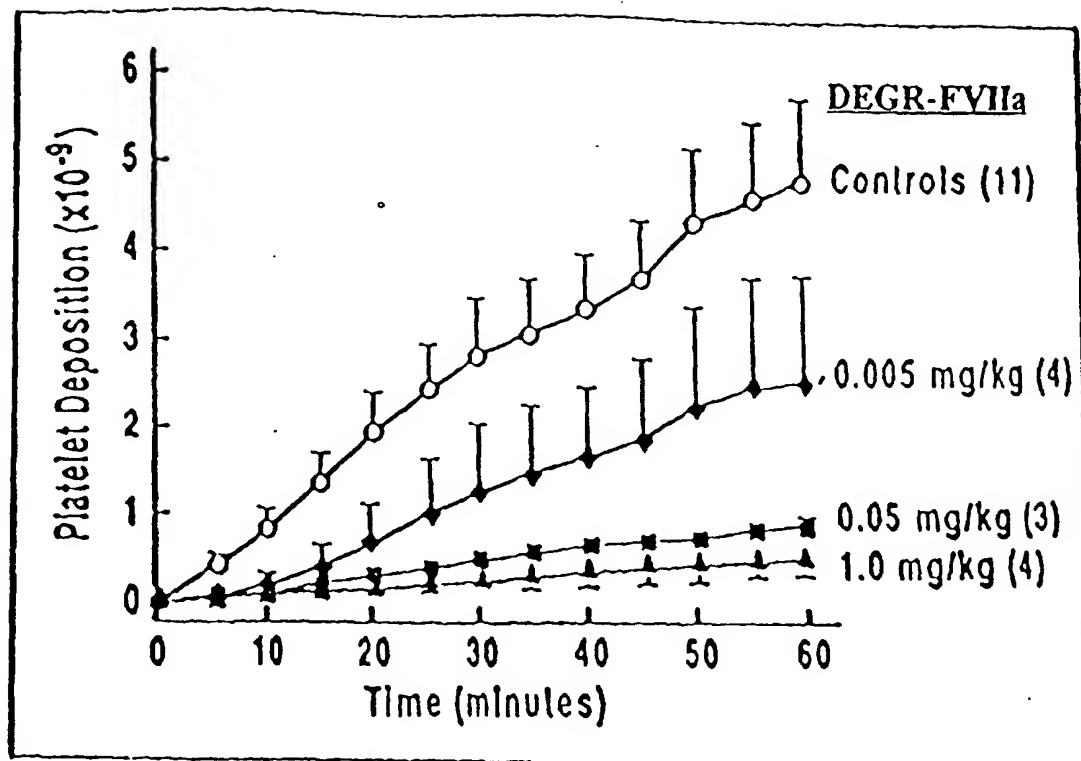
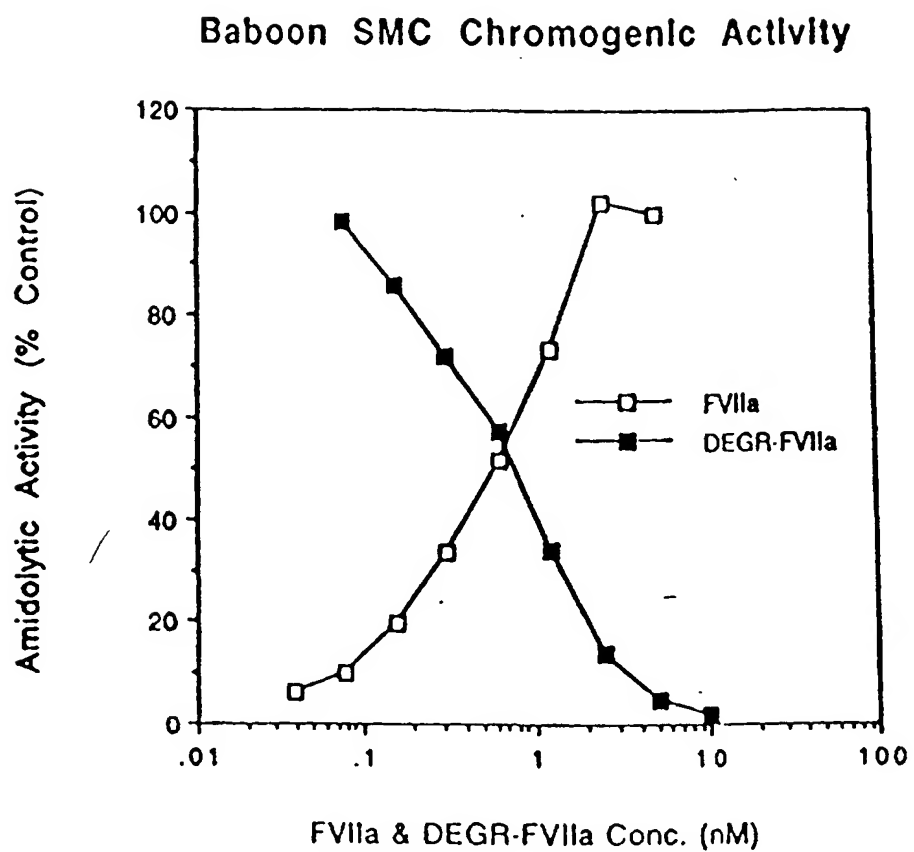


Fig. 2

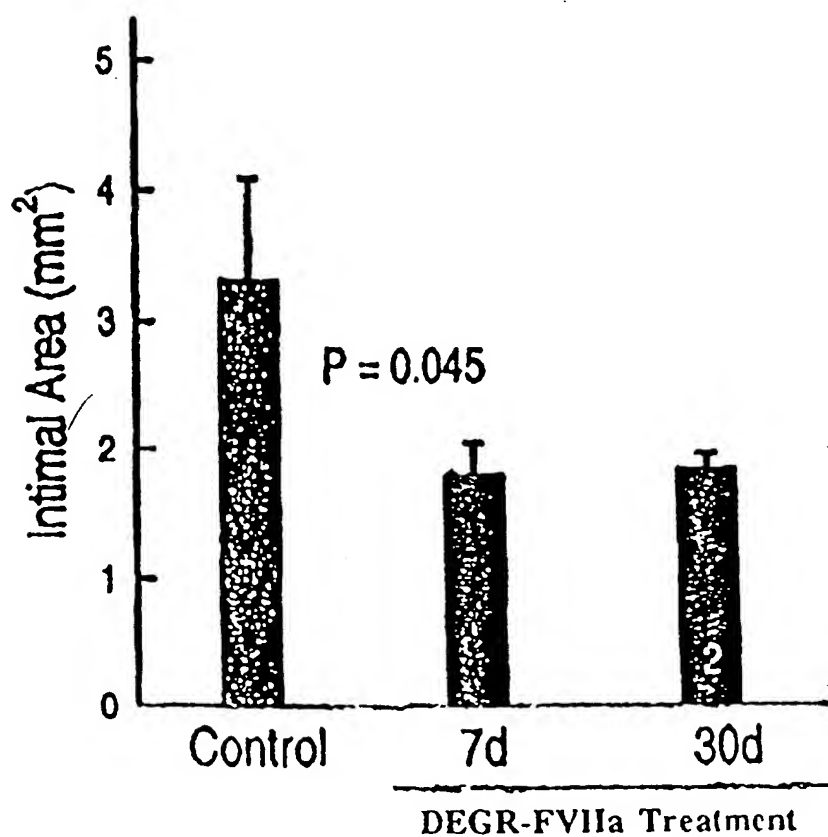
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Fig. 3



4/11

Fig. 4

**Carotid Artery Endarterectomy**



5/11

## Femoral Artery Balloon Injury

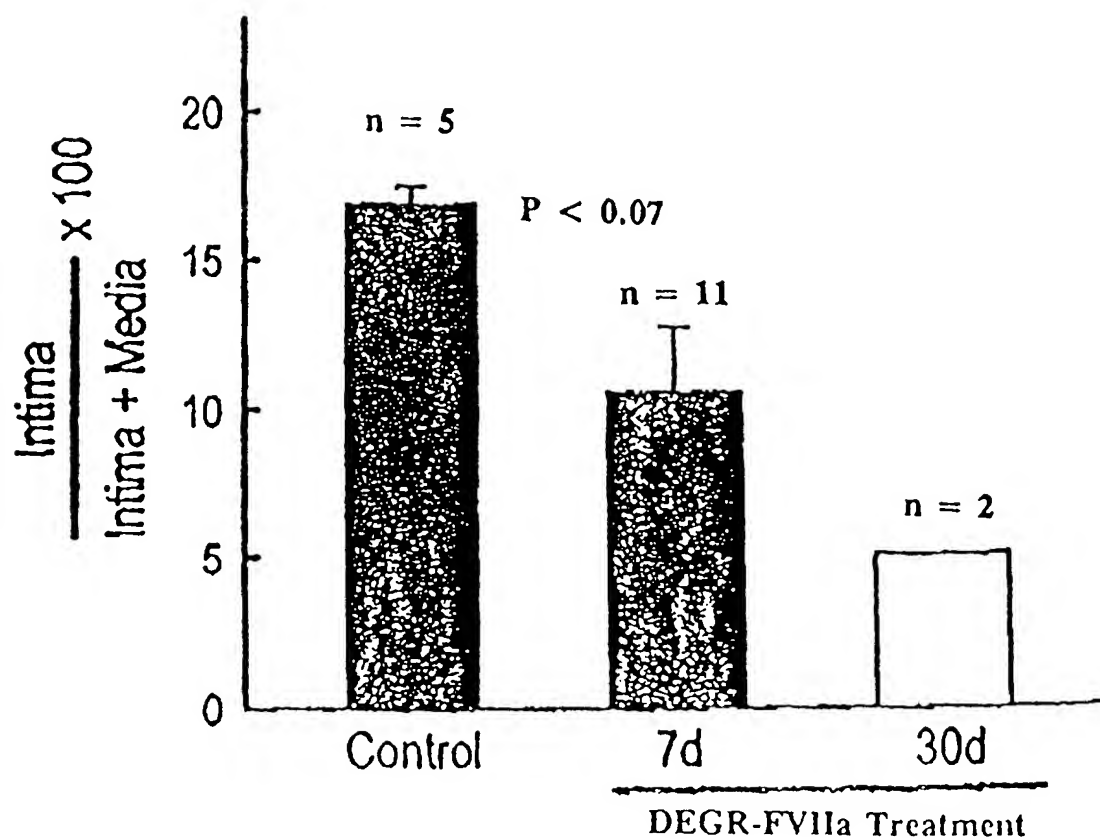


Fig. 5

6/11

# EXPERIMENTAL PROTOCOL

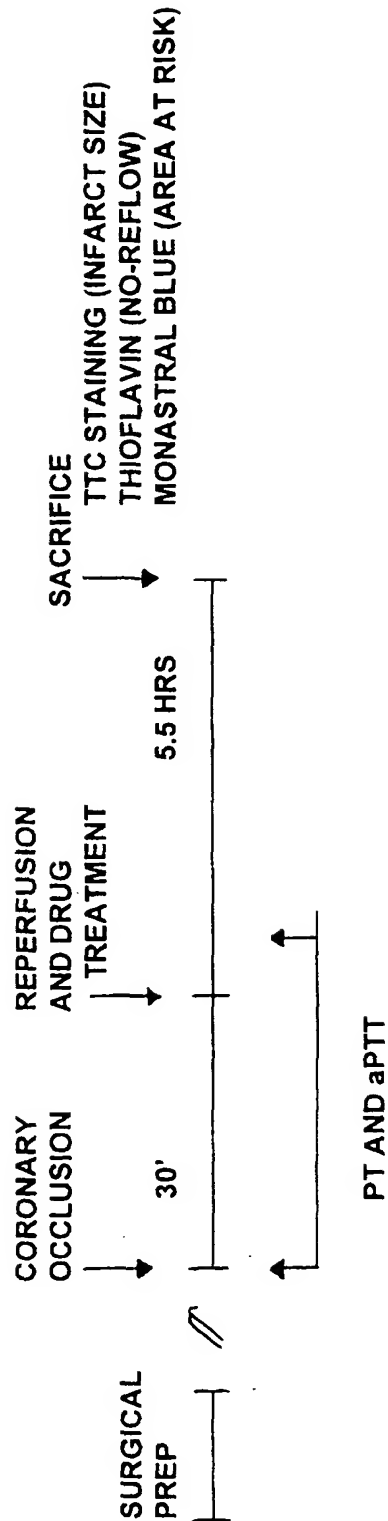


Fig. 6

7/11

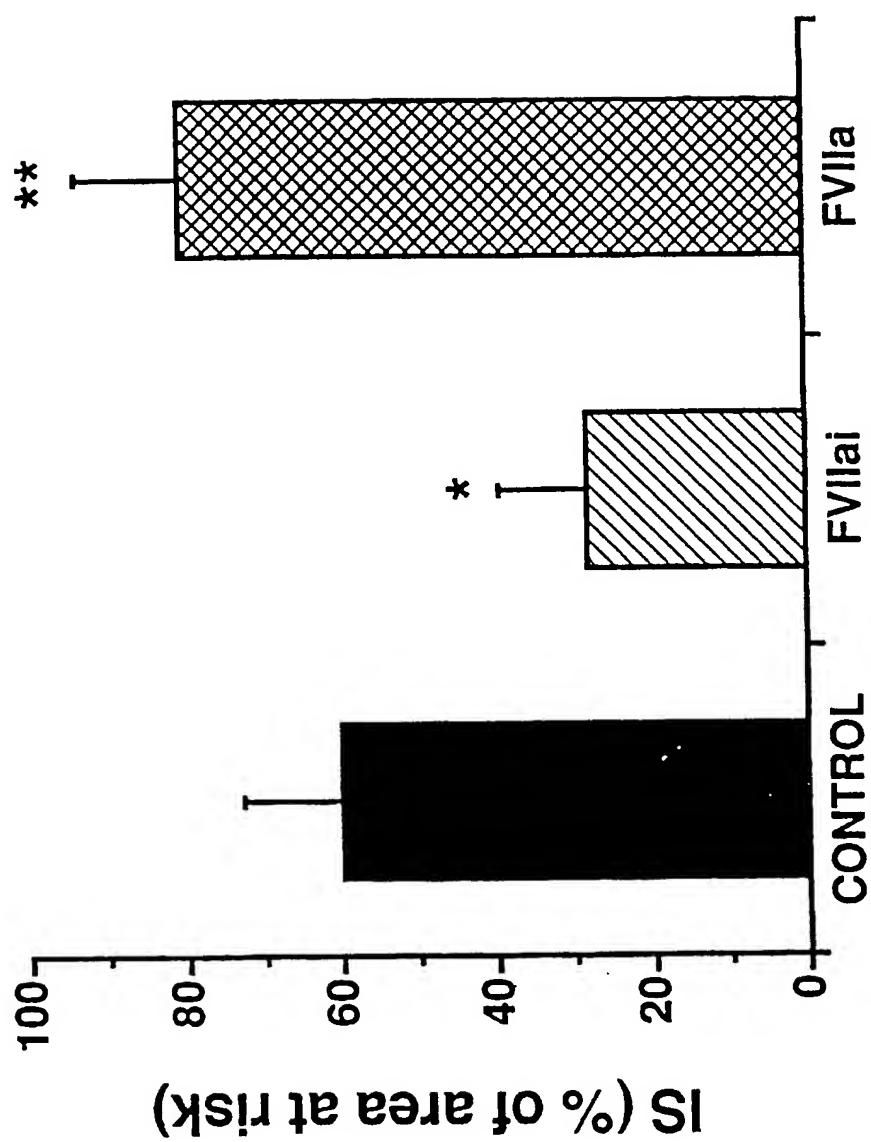


Fig. 7

8/11

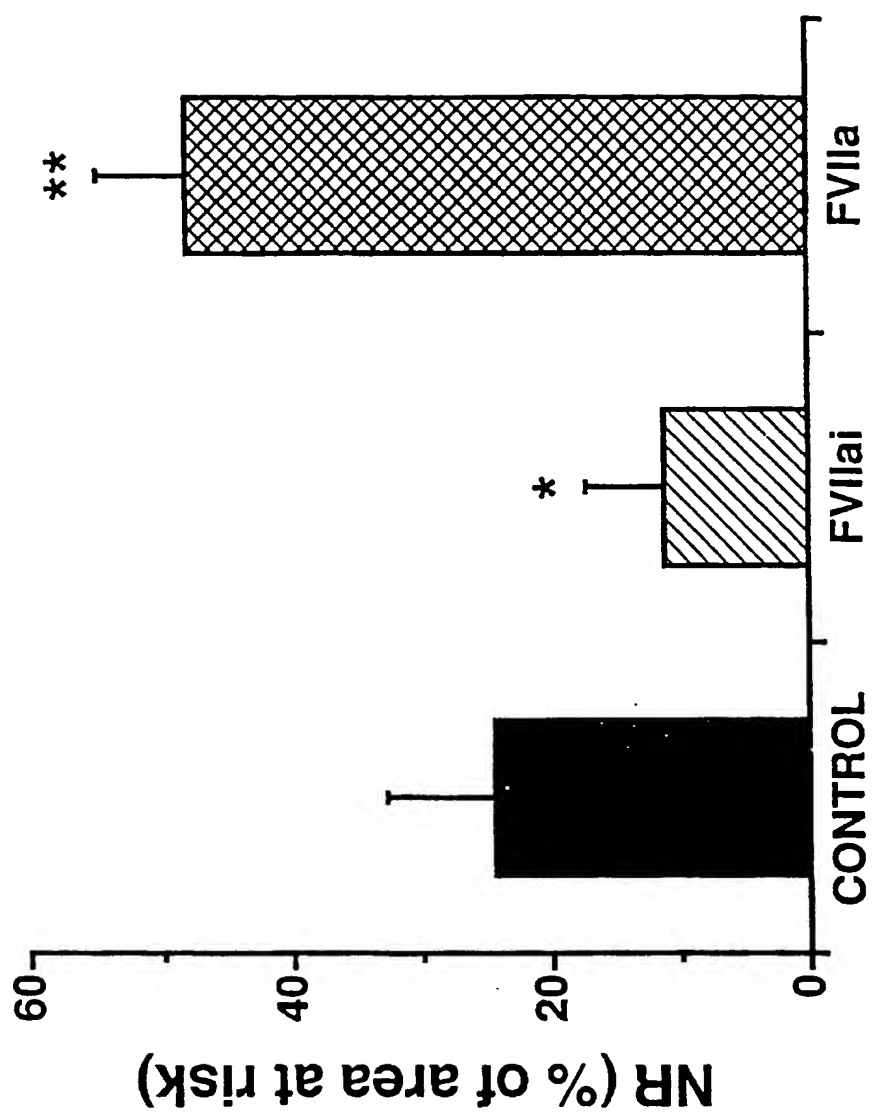


Fig. 8

9/11

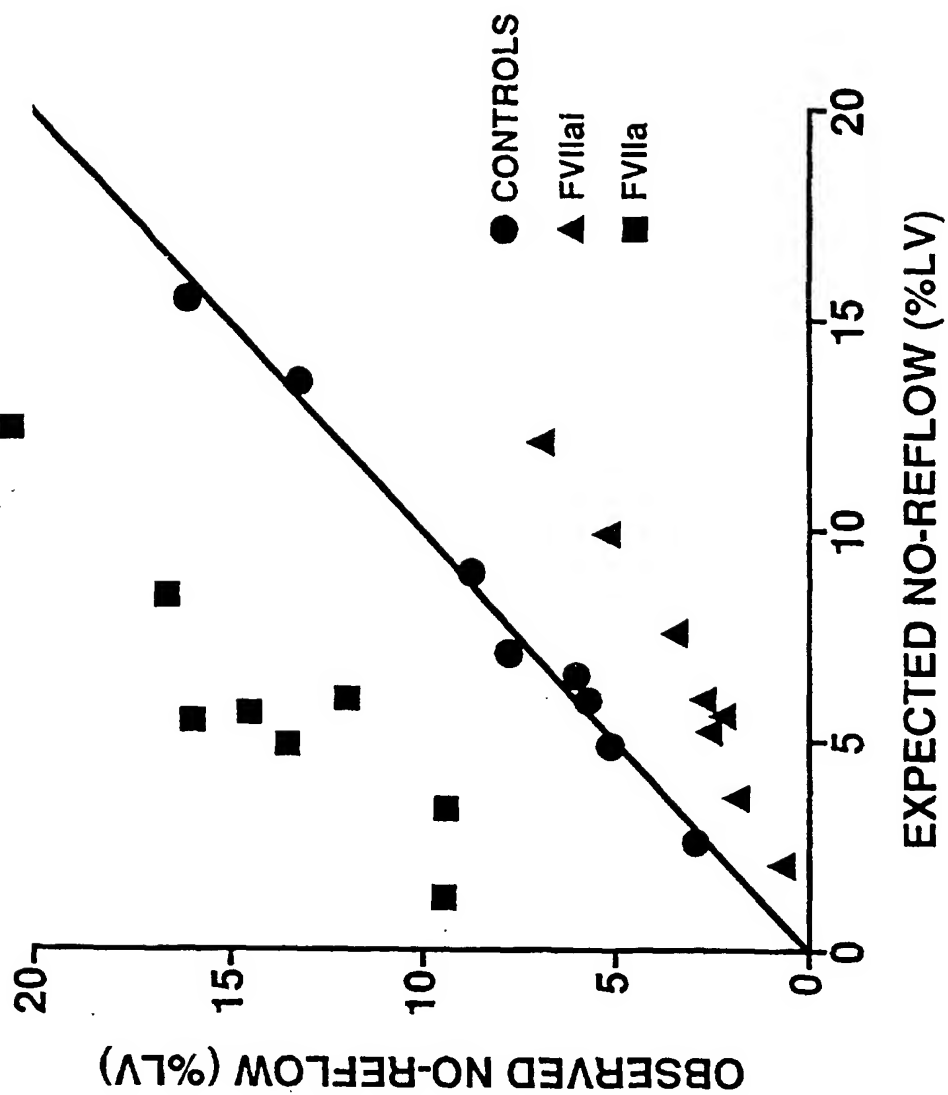


Fig. 9

10/11

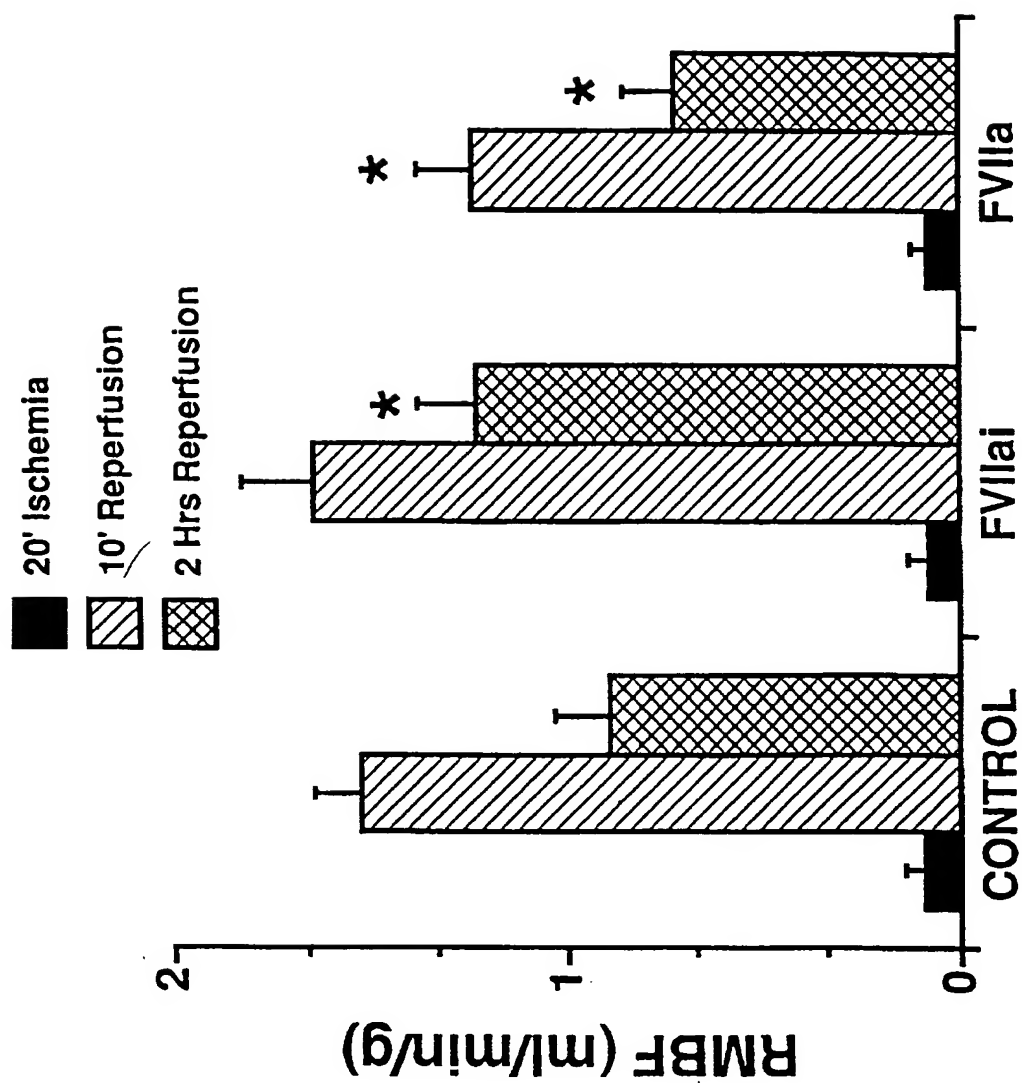


Fig. 10

11/11

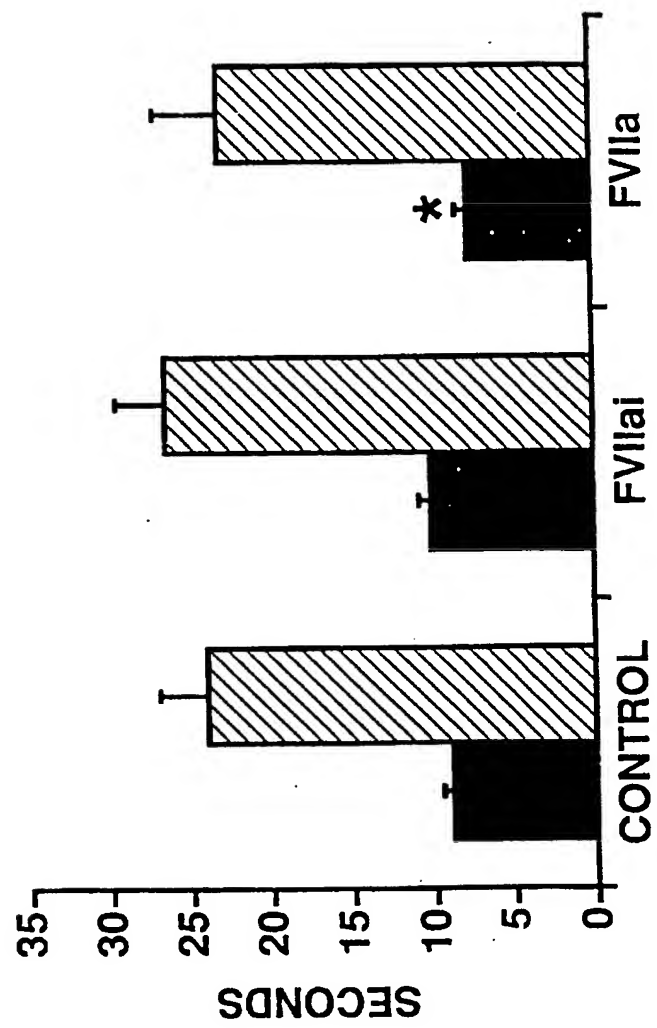


Fig. 11

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00251

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>		
IPC6: C07K 14/745, A61K 38/36 According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols)		
IPC6: C07K, A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
SE,DK,FI,NO classes as above		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
WPI, US PATENTS FULLTEXT, MEDLINE, BIOSIS, EMBASE		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9215686 A1 (ZymoGenetics, Inc.), 17 Sept 1992 (17.09.92), page 4, line 9 - line 11; page 5, line 5; page 7, line 6 - line 16, page 14, line 18 - line 28; page 15, line 14 - line 21; page 15, line 27, claims 1-26 --	1-28
X	WO 9427631 A1 (ZymoGenetics, Inc.), 8 December 1994 (08.12.94), the claims --	1-28
A	WO 9606637 A1 (Washington University), 7 March 1996 (07.03.96), page 1, line 6; page 2, line 26 - page 3, line 27; page 4, line 23 - line 24, page 5, line 11 - line 14; page 6, line 15 - line 16; page 10, line 18 - line 20 -- -----	11-28
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "B" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
1 Sept 1997		04 -09- 1997
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86		Authorized officer  Carolina Palmcrantz Telephone No. +46 8 782 25 00



**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/DK 97/00251

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-10, 16-20, 25-28  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 1-10, 16-20, 25-28 are directed to methods of treatment of the human/animal body the search has been carried out and based on the alleged effects of the composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐

The additional search fees were accompanied by the applicant's protest.

☐

No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

06/08/97

International application No.

PCT/DK 97/00251

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9215686 A1	17/09/92	AU 672357 B	03/10/96
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		CA 2103546 A	29/08/92
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		US 5648331 A	15/07/97